Neonatal enteral feeding tube as loci for *Enterobacteriaceae* colonisation and risk to neonatal health

by

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STATEMENT

I hereby certify that all the research work presented here in this thesis is the result of my own work except where the references have been made to the published literature or previous research work.

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Abstract

The incidence of neonatal infections caused by *Enterobacteriaceae* has been increasing in recent years, and they are now recognised as the predominant causative agents in neonatal intensive care unit (NICU) outbreaks. *Klebsiella* spp. and *Serratia* spp. are the most common causative pathogens, and *E. coli* is one of the leading causes of neonatal meningitis and sepsis. The infant intestinal flora is influenced by the feeding regime. This study focuses on assessing the risk to neonates from the ingestion of the *Enterobacteriaceae* such as; *Enterobacter hormaechei*, *Enterobacter ludwigii*, *Enterobacter aerogenes, Enterobacter cloacae* and *Klebsiella oxytoca.* The strains under study were isolated from two sources; human mastic breast milk (MBM) and neonatal nasogastric enteral feeding tubes (EFT).

The overall aim was to evaluate the risk to neonates posed by the ingestion of these organisms either from contaminated breast milk or from infant formula.

Due to the lack of adequate source information, it was necessary to first confirm the identity of the strains under investigation. This was achieved using standard biochemical profiles (phenotyping) and where necessary 16S rDNA sequence analysis. Secondly, it was necessary to determine whether all strains were unique or if any were multiple isolations of the same strain. This was achieved using Pulsed-Field Gel Electrophoresis (PFGE). To determine the potential exposure of neonates to these organisms, a range of physiological and virulence related assays were undertaken; heat tolerance to 55°C, biofilm formation, capsule formation and acidic pH survival (pH 3.5).

The potential virulence of the strains was assessed using attachment-invasion assays of human Caco-2 intestinal cells, human brain microvascular endothelial cells (HBMEC) and rat brain capillary endothelial cell line (rBCEC4); and also persistence of bacteria in macrophages by using U937 cells. Patterns of adherence of *Enterobacteriaceae* to Caco-2 cells was investigated. The presence of the virulence factors of strains was determined by identifying haemolytic activity, serum resistance, siderophore production and antimicrobial susceptibility. The iron uptake genes were also investigated.

The results by PFGE showed that neonatal enteral feeding tubes and mastic human breast milk were contaminated by twenty-one and three pulsotypes of *Enterobacteriaceae*, respectively. Furthermore, the same pulsotypes were spread among enteral feeding tubes of infants in the same NICUs; indicating the same origins, such as: environment, milk or carer. Similarly, the MBM strains were isolated from the same mother. The identification of strains by using 16S rDNA sequence analysis (genotyping) was more accurate than phenotyping (API technique) and the clustering of strains by PFGE is a suitable technique for strains relatedness.

The physiological features of the strains in the current study were investigated. The ability of strains to survive at 55ºC was studied and most of the strains were able to survive at 55ºC for >30 minutes. Biofilm formation was investigated as this may be a factor of organism persistence in the neonatal intensive care unit (via milk, environment or workers) and attachment to enteral feeding tubes. All strains formed biofilms and this was, in general, enhanced at 37°C compared with room temperature (20ºC) in all types of formula. The highest levels of biofilm were in casein-based infant formula. Most strains produced capsular material at 37ºC on all types of formula. However, capsular material was produced by all strains in soya infant formula. All strains were able to survive at pH 3.5 for up to 2 hours.

All strains were able to attach to Caco-2, HBMEC and rBCEC4 cells lines, while there was variation between strains ability to invade mammalian cells. In particular, most of *Ent. hormaechei* strains were able to invade the three types of cells lines and one *Ent. ludwigii* strain 1439 was only able to invade the rBCEC4 cell line. *Ent. ludwigii* strain 1439 was isolated from a case of neonatal meningitis. Three out of eight strains of *Ent. hormaechei* and two strains of *Ent. cloacae* strains survived within macrophages. Haemolysin production, serum resistance and siderophore production were also studied and all strains were positive.

Genes encoding for iron uptake *irp1, irp2* and *fyuA* were detected whereas *irp1, irp2* genes were absent in all strains while *fyuA* was present in 4/6 of *Ent. ludwigii* strains, 1/8 of *Ent. hormaechei*, 2 of *Ent. cloacae*, 1 of *Ent. aerogenes* and 3/6 of *K. oxytoca*. Three

out of eight strains of *Ent. hormaechei* showed resistance to even the 3rd generation cephalosporins, ceftazidime and cefotaxime and were ESBL-positive.

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DEDICATION

This thesis is lovingly dedicated to the memory of my father who will always be missed. Special feeling of gratitude to my mother Sharefa, who taught me that even the most difficult of tasks can be accomplished with determination. It is also dedicated to my lovely sisters without whose encouragement and support, I would not have completed this work.

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ABBREVIATIONS LIST

A.	*Acinetobacter*
Ae.	*Aerobacter*
Aer.	*Aeromonas*
BP	Base pairs
Caco-2	Human colonic carcinoma epithelial cells
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
Cit.	*Citrobacter*
Cl.	*Clostridium*
COSHH	Control Of Substances Hazardous to Health
CPD	Combination of cefpodoxime
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleoside 5'-triphosphate
dNTPs	Deoxyribonucleotide Triphosphate
E.	*Escherichia*
EDTA	Ethylenediamine tetra-acetic acid
EFT	Enteral feeding tubes
EHOS	Ent. hormaechei outbreaks
Ent.	*Enterobacter*
ESBLs	Extended spectrum β-lactamases
EPS	Extracellular polymeric substances
FAO	Food and Agriculture Organization
HBMEC	Human brain microvascular endothelial cells
HCL	Hydrochloric acid
HPA	Health Protection Agency
ICUs	intensive care units
ISO	Iso-Sensitest agar
K.	*Klebsiella*
KAH	King Abdulla Hospital

LBA	Luria-Bertani agar
LBA	Luria-Bertani Agar
LPS	Lipopolysaccharide
MBM	Mastic breast milk
Mo.	*Morganella*
MOI	Multiplicity of infection
MRSA	Methicillin-Resistant Staphylococcus aureus
My.	*Mycobacterium*
NCH	Nottingham City Hospital
NICU	Neonatal intensive care unit
NNIS	National nosocomial infections surveillance system
NTU	Nottingham Trent University
OD	Optical Density
P.	*Pseudomonas*
PBS	Dulbecco's phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PIF	Powdered infant formula
Pl.	*Plesiomonas*
Pr.	*Providencia*
PRH	Princesses Rahma Hospital
QMC	The Nottingham Queen's Medical Centre
R	Resistance
rBCEC4	Rat brain capillary endothelial cell line
rpm	Revolutions per minute
S	Sensitive
S.	*Streptococcus*
Sal.	*Salmonella*
SDS	Sodium dodecyl sulphate
Se.	*Serratia*
Sh.	*Shigella*

Spp.	Sepsis
St.	*Staphylococcus*
TAE	Tris-acetate-EDTA
TBE	Tris base- Boric acid – EDTA
TEB	Tris EDTA buffer
TIFF	Tagged Image File Format
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
U	Unique
U937	Macrophage Cell Line
UN	United Nations
UPGMA	Unweight pair group method with arithmetic mean
US	United States
USA	The United States of America
UV	Ultraviolet
V.	*Vibrio*
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
Y.	*Yersinia*
A	Alpha
B	Beta
γ	Gamma

Chapter 1: INTRODUCTION

1.1 INTRODUCTION

The *Enterobacteriaceae* is a family of Gram-negative bacilli, some of which are motile, and have capsules. Most species are facultative anaerobic, oxidase-negative and catalase-positive rods. The *Enterobacteriaceae* which most commonly produce extended-spectrum β-lactamases (ESBLs) are *Klebsiella pneumoniae* and *Escherichia coli* (Romero *et al*., 2007). Some members are found in soil, water, plants and animals. The *Enterobacteriaceae* group is comprised of more than 100 species of bacteria that normally inhabit the intestines of humans and animals. Many *Enterobacteriaceae* species are lactose fermenting and are commonly referred to as "coliforms". Exotoxins are produced by some pathogenic strains, whereas others produce enterotoxins which affect the intestinal tract, causing diarrhoea and body fluid loss. Some *Enterobacteriaceae* (i.e. *K. pneumoniae*) are able to cause pneumonia and urinary tract infections. Wound infections and other nosocomial (hospital acquired) infections are caused by *Enterobacteriaceae*. They are also recognized as a major cause of bacteraemia and meningitis.

In recent years, the incidence of neonatal infections due to *Enterobacteriaceae* has been increasing and they are now recognised as the predominant causative agents of infection in neonatal intensive care unit (NICU) outbreaks (Gastmeier *et al*., 2007; McGuire *et al*., 2004; Kaufman *et al*., 2004). The most common neonatal *Enterobacteriaceae* pathogens reported are *Klebsiella* spp. and *Serratia* spp.; 23.4% and 13.8% cases respectively (Gastmeier *et al*., 2007). There are also other pathogenic species which, although they occur less frequently, have a high severity following infection. For example, pathogenic strains of *E. coli* are a leading cause of neonatal meningitis and sepsis (Stoll *et al*., 2005), and *Cronobacter sakazakii* can cause necrotizing enterocolitis and meningitis. Neonates may be particularly prone to Gram negative infection (i.e. *Enterobacteriaceae*) as their innate immune cells have lower responses to lipopolysaccharide (LPS), the outer portion of the bacterial cell wall (Townsend *et al*., 2007). However, Nanthakumar *et al.* (2000) reported that the human colonic carcinoma epithelial (Caco-2) cells secrete less IL8 (LPS, 8-fold; IL-1b, 20-fold) than fetal cells after inflammatory stimulation.

1.2 Impact of *Enterobacteriaceae* on neonates

Enterobacteriaceae are the most common opportunistic pathogens particularly *E. coli*, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Salmonella*. They are significantly implicated with morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012). Many different types of infections can be caused by these organisms, such as sepsis, brain meningitis, pneumonia, and urinary tract infections particularly in intensive care units (ICUs) (Kollef *et al*., 1999; Ibrahim *et al*., 2000). *Enterobacteriaceae* such as *E. coli, Klebsiella*, *Proteus, Serratia* and *Citrobacter* spp. are able to cause nosocomial infections. Most of these strains are members of normal flora such as *E. coli* and *Ent. cloacae,* and probably cause infections through selection following an empiric antimicrobial regime (Iversen *et al*., 2004a; Stoll *et al*., 2005). The most common human diseases which are caused by a family of *Enterobacteriaceae* are presented in Table 1.1 (Liu *et al*., 2012; Podschun and Ullmann, 1998; Pazhani *et al*., 2005; Fraser and Arnett, 2006; Chaudhry *et al*., 2007; Hammerum and Heuer, 2009; Bisi-Johnson *et al*., 2011).

Table 1.1: Most common species *Enterobaceriaceae* causing clinical infectious disease.

Clinical species	Clinical Infection Presentation
Cit. freundii	Pneumonia, meningitis, septicaemia, wound and urinary tract infections
Ent. aerogenes, Ent. cloacae	Pneumonia, septicaemia, wound and urinary tract infections
E. coli	Diarrhoea, meningitis, septicaemia and urinary tract infections
K.oxytoca, K. pneumoniae	Pneumonia, septicaemia and urinary tract infections
Mo. morganii	Septicaemia and urinary tract infections
Pl. shigelloides	Diarrhoea and septicaemia
Pr. rettgeri, Pr. stuartii	Urinary tract infections
Sal. enteritica	Diarrhoea, typhoid fever, septicaemia, osteomyelitis and urinary tract infections
Se. marcescens, Se. liquefaciens	Pneumonia, septicaemia, wound and urinary tract infections
Sh. sonnei, Sh. flexneri	Diarrhoea
Y. pestis, Y. enterocolitica	Diarrhoea, septicaemia plague and enteritis

1.3 *Enterobacter* spp. as a cause of neonatal infection

The *Enterobacter* genus belongs to the family of *Enterobacteriaceae* and are known as facultative anaerobic Gram-negative strains. This genus are saprophytic bacteria which

are widely encountered in nature, such as in soil and sewage and are commensal in the enteric flora in the human gastrointestinal tract (Mazari-Hiriart *et al*., 2008; Quintanilha *et al.,* 2007). There are 14 known species of *Enterobacter*, some of these have been implicated as causes of diseases in humans and the most commonly encountered species are *Ent. aerogenes*, *Ent. cloacae*, *Ent. agglomerans*, and *C. sakazakii* (Andresen *et al*., 1994; Burchard *et al*., 1986; Chow *et al*., 1991; Gallagher 1990; Gaston 1988; Haddy *et al*., 1991; Hawkins *et al*., 1991; Stenhouse *et al*., 1992).

Ent. aerogenes and *Ent. cloacae* are considered the most frequently encountered human pathogens within the genus *Enterobacter* (Andresen, *et al*., 1994; Burchard *et al*., 1986; Haddy *et al*., 1991; Burchard *et al*., 1994; Karnad *et al*., 1987; Weischer and Kolmos, 1992). The major nosocomial pathogens often found in intensive care settings are *Enterobacter* species. From 1976 to 1989 in the United States of America (USA), the National Nosocomial Infections Surveillance System (NNIS) reported data on nosocomial bacteremia. In 2008 the National Healthcare Safety Network reported that *Enterobacter* spp. accounted for approximately 5% of nosocomial bacteremia cases, between 1995 and 2002, a study by Wisplinghoff *et al*. (2004) from different states in USA demonstrated the most commonly isolated nosocomial pathogens with a higher rate in intensive care unit wards was *Enterobacter* species (Wisplinghoff *et al*., 2004). Previous studies by v Dijk *et al.* (2002) and van den Berg *et al.* (2000) reported that neonatal intensive care units (NICUs) have outbreaks of *Enterobacter* spp.

1.3.1 *Ent. cloacae*

Ent. cloacae are members of the genus *Enterobacter*. As other *Enterobacteriacae*, they are saprophytic microorganisms of the normal digestive flora in humans and are the most commonly isolated clinical species (Thomas *et al*., 1993; Wang *et al*., 1991). Recently, six species have been classified in the *Ent. cloacae* complex, including *Ent. cloacae*, *Ent. asburiae*, *Ent. hormaechei*, *Ent. kobei*, *Ent. ludwigii* and *Ent. nimipressuralis*. The biochemical and molecular studies on *E. cloacae* have shown genomic heterogeneity, comprising six species. Species of the *Ent. cloacae* complex are widely encountered in nature, but they can act as pathogens (Mezzatesta *et al*., 2012).

Ent. cloacae has emerged as an alarming pathogen for healthcare institutions globally. It accounts for up to 5% of hospital-acquired sepsis, 5% of nosocomial pneumonias, 4% of nosocomial urinary tract infections and 10% of postsurgical peritonitis cases (Paauw *et al*., 2008a and Hoffmann and Roggenkamp, 2003). In different clinical settings, including neonatal NICUs, outbreaks due to exogenous *Ent. cloacae* infection have been reported. In 1998, van Nierop *et al*. reported an outbreak of *Ent. cloacae* in a NICUs that caused nine deaths. Also, Kuboyama *et al.* (2003) reported an overall 34% mortality rate associated with *Ent. cloacae* in unrecognized outbreaks in NICU. This bacterium was possibly transmitted to neonates by contaminated intravenous fluids, whole parenteral nutrition solutions and medical equipment.

The healthcare workers may play a role in cross-transmission of many single-clone outbreaks and inpatients may also act as a reservoir (Dalben *et al*., 2008). In hospitalized neonates, *Ent. cloacae* can cause a range of infections such as urinary tract infection, bloodstream infection, pneumonia and bronchopulmonary dysplasia (Grattard *et al*., 1994; Modi *et al*., 1987). *Enterobacteriaceae* has been isolated from powder infant formula (PIF), such as *Ent. cloacae*, *K. pneumoniae*, *K. oxytoca*, *Ent. hormaechei*, *Cit. freundii*, and *E. coli* (Muytjens *et al*., 1988; Townsend *et al*., 2008a).

Only a few of the studies have reported enteral feeding tubes (EFT) as a site of the bacterial colonisations. Bacterial contamination of the EFT with $>10^3$cfu/ml in 71/125 EFTs was reported by Mehall *et al*. (2002a) from infants over four months of age. *Staphylococcus epidermidis*, *S. aureus*, *Enterococcus faecalis*, *Ent. cloacae* and *K. pneumoniae* were detected in their study. They also showed that seven infants older than four months who were fed with tubes containing $>10^5$cfu/ml developed necrotizing enterocolitis. Their study also identified methicillin-resistant *Staphylococcus aureus* (MRSA) contamination in the EFT (Mehall *et al*., 2002b).

1.3.2 Ent. hormaechei

Ent. hormaechei is a Gram-negative rod in the *Ent. cloacae* complex, and is most frequently isolated from clinical sources (Townsend *et al*., 2008a). The species have been defined within the *Ent. cloacae* complex via deoxyribonucleic acid (DNA) cross-

hybridization, naming three new subspecies of *Ent. hormaechei*: *Ent. hormaechei* subsp. *steigerwalti, Ent. hormaechei* subsp. *hormaechei* and *Ent. hormaechei* subsp. *oharae* (Hoffmann *et al.*, 2005a).

Using biochemical identification, *Ent. hormaechei* strains are catalase positive, oxidase and deoxyribonuclease (DNase) negative and fermentative. *Ent. hormaechei* form non-pigmented colonies after 18 to 24 hours at 15 to 42°C, with an optimum for growth at 36°C, on all non-selective media, such as Colombia agar with 5% sheep blood, chocolate agar, tryptic soy agar (TSA), Luria-Bertani agar (LBA), and brain heart infusion agar, as well as on semi-selective media such as MacConkey and ENDO agar (Hoffmann and Roggenkamp, 2003).

Ent. hormaechei is the most commonly isolated nosocomial pathogen (Paauw *et al*., 2008a). It has been a cause of septicaemia in an outbreak occurring in six NICUs in five hospitals in Brazil, and due to contamination of parental nutrition solutions which all originated from the same manufacturer (Campos *et al*., 2007). Ciprofloxacin resistance was closely associated with the prior use of fluoroquinolones and broad-spectrum cephalosporin in *Enterobacter* species isolates causing bacteremia (Kang *et al*., 2005).

A previous study conducted by the Post-doctoral researcher Dr Stacy Townsend at Nottingham Trent University (NTU) identified *Ent. hormaechei* from isolates received from a United States (US) hospital that had been misidentified as 'Cronobacter' strains. Further analysis, with Pulsed Field Gel Electrophoresis (PFGE) and 16S rDNA sequencing revealed that the strains were clonal and were from an NICU outbreak which the hospital had not detected (Townsend *et al*., 2008a). Hence, the hospital had not only failed recognising that they had an outbreak, but had also misidentified the organisms.

Muytjens *et al*. (1988) identified *Cronobacter* strains from PIF using biochemical profiling. However, by using 16S rDNA sequence analysis some of the strains were reidentified as *Ent. hormaechei*. This organism has been reported to be resistant to quinolone highlighting the difficulty of treatment (Townsend *et al*., 2008a). The study of this organism is significant in the clinical setting as it is difficult to treat and identify. *Enterobacteriaceae* isolates identified from EFT from the Nottingham Queen's Medical

Centre (QMC) and Nottingham City Hospital (NCH) included *Ent. hormaechei* (Hurrell *et al*., 2009b). Many of these strains were resistant to the 3rd generation cephalosporins, ceftazidime and cefotaxime, and ESBLs were detected. This demonstrates that neonates are directly exposed to antibiotic resistant strains in NICUs during feeding.

Comparative bacterial thermal death rates of *Enterobacteriaceae* revealed that an *Ent. hormaechei* strain isolated from a neonatal enteral feeding tube was more heat resistant that *Sal. seftenberg*. This latter organism is recognised as the most heat resistant *Enterobacteriaceae* and its detection is used by industry to ensure their heat treatment processes are efficient (Forsythe, 2009). This shows that *Ent. hormaechei* has a greater chance of surviving during the reconstitution of infant formula than other organisms, and that preparation may need to be modified to take this into account.

1.3.3 Ent. aerogenes

Ent. aerogenes is known as part of the normal flora of the human gastrointestinal tract that is in most cases endogenously acquired (Flynn *et al*., 1987). In addition, *Ent. aerogenes* is an opportunistic colonizing pathogen which can cause infection in hospitalized patients (Sanders and Sanders, 1997). This organism is one of the more commonly described Gram-negative bacteria causes of nosocomial respiratory tract infections (Arpin *et al*., 1996; Davin-Regli *et al*., 1996a). Recently, *Ent. aerogenes* has emerged as a significant hospital pathogen (de Champs *et al*., 1991; Flynn *et al*., 1987; Mellencamp *et al*., 1990; Meyers *et al*., 1988). Because of the widespread prescription of broad-spectrum antibiotics, especially extended-spectrum cephalosporins, *Ent. aerogenes* has emerged as a pathogen (Shlaes, 1993).

The presence of medical devices such as endotracheal tubes or central venous catheters are associated with multiresistant *Ent. aerogenes*, which facilitate colonization (Chow *et al*., 1991; Weinstein, 1986).

Ent. aerogenes has been associated with nosocomial infections during the last 5 years. Several outbreaks linked with multiresistant strains of *Ent. aerogenes* have been reported in ICUs in France (Arpin *et al*., 1996; Davin-Regli *et al*., 1996 a and b; Grattard *et al*.,

1995; Neuwirth *et al*., 1996), Belgium (De Gheldre *et al*., 1997; Jalaluddin *et al*., 1998), Austria (Allerberger *et al*., 1996) and the United States (Georghiou *et al*., 1995).

Ent. aerogenes species are not commonly isolated in NICUs however, a previous study by Gallagher (1990) reported that in a pediatric population during a 5 year period, 22 episodes of nosocomially acquired *Enterobacter* bacteria were identified: 17 were *Ent. cloacae*, three were *Ent. aerogenes* and two were *Ent. agglomerans*. Another study also reported that *Ent. aerogenes* was isolated in a NICU from the blood culture of a 5 day old neonate; and 12 more episodes were found 10 weeks later which caused nosocomial outbreaks of septicemia in the NICU (Loiwal *et al*., 1999).

1.3.4 *Ent. ludwigii*

Ent. ludwigii is a Gram-negative rod, belonging to the *Enterobacteriaceae* family, and is motile, catalase positive, oxidase and DNase negative, fermentative, and non-pigmented. *Ent. ludwigii* has the ability to ferment 3-0-methyl-D-gluco-pyranose and myo-inositol. *Ent. ludwigii* has been reported to produce a Bush class 1 beta-lactamase rendering resistance to ampicillin, amoxicillin plus clavulanic acid and cefoxitin in the disk diffusion tests. In addition, *Ent. ludwigii* is susceptible to cefepime, trimethoprim plus sulphamethoxazole, gentamicin, and resistant to piperacillin, piperacillin plus tazobactam, cefoxitin, cefotaxime, ceftazidime (Hoffmann *et al*., 2005b).

There have been few studies of this organism, possibly due to the lack of a reliable identification scheme. However, during the second year of this project, we received an additional *Ent. ludwigii* strain (NTU1439) from Nottingham QMC hospital which had been isolated from a case of neonatal meningitis. This strain was included in the project as it reinforced the importance of evaluating the risk of neonatal infection by *Ent. ludwigii*.

In previous studies, *Ent. ludwigii* has been isolated from various sources such as clinical specimens (Hoffmann *et al*., 2005b), and from plants (Shoebitz *et al.*,2009). Delgado *et al*. (2008) isolated *Ent. ludwigii* from human milk. However, as will be reported here, it was originally identified as *K. oxytoca* using classical tests. By using 16S rDNA sequence analysis, the Delgado *K. oxytoca* isolates were reidentified as *Ent. ludwigii* at

NTU. By coincidence, NTU already had an *Ent. ludwigii* strain in its collection which was initially identified as *Cronobacter;* this strain isolated from a case of neonatal meningitis in Nottingham QMC hospital.

1.4 *Klebsiella* spp. as a cause of neonatal infection

The most commonly isolated members of the genus *Klebsiella* (family *Enterobacteriaceae*) are *K. pneumoniae* and *K. oxytoca* which consist of Gram-negative, non-motile, encapsulated rods (Green *et al*., 2009), and the colonies of *Klebsiella* have a characteristic mucoid appearance because they produce polysaccharide capsules (Podschun and Ullmann, 1998). *Klebsiella* spp. are opportunistic pathogens that can cause a variety of illnesses including pneumonia, urinary tract infections, septicaemia, soft tissue, intravenous, meningitis, liver abscess, and gastrointestinal disease common in immunocompromised or those with underlying conditions (Green *et al*., 2009).

To cause infection, microorganisms must adhere to the mucosal surfaces in order to reach host cells and can use surface appendages to achieve this (Podschun and Ullmann, 1998). *Klebsiella* is abundant in the environment (e.g., soil and water) but can also be isolated from skin, mucous membranes, and the intestines of humans and animals (Podschun and Ullmann, 1998). In a study of bacterial colonisation in a NICU, there was a higher risk of the acquisition of *Klebsiella* spp., and *Enterobacter* spp., in infants receiving antibiotics for more than 3 days and associated with an increased duration of NICU stay. *Klebsiella* spp. is recognised to survive on the skin and is more resistant to desiccation than other *Enterobacteriaceae* (Gastmeier *et al*., 2007).

1.5 Influence of the existing gut microbiome on neonates health

Colonization of the human gut with microbes begins during birth by microbes from the environment. In the first few hours of life, usually the most important source of inoculum is the mother's vaginal and fecal microbiomes (Gueimonde *et al*., 2006 and Vaishampayan *et al*., 2010). However, some data reported that infants delivered by C-section-delivered have lower gut microbial richness and variety at 4 months of age,

compared to vaginally delivered infants (Azad *et al*., 2013). The great functional and phylogenetic difference observed between infant gut microbiomes possibly because of random colonization happenings, variances in immune responses to the colonizing microbes, alterations in host behaviour, or other features of host lifestyle (Palmer *et al*., 2007; Dethlefsen *et al*., 2006).

The previous studies report that the microbiome is implicated in human health (Turnbaugh *et al*., 2007). Gut microbiotas can contribute to excess host adiposity (Turnbaugh and Gordon 2009; Turnbaugh *et al*., 2006), protect against the improvement of type 1 diabetes (Wen *et al*., 2008), and induce colitis (Garrett *et al*., 2007) and metabolic syndrome (Stockman, 2012). Hence, the microbiota has been proposed as a target for therapeutic intervention for numerous chronic diseases (Turnbaugh *et al*., 2007; Zaneveld *et al*., 2008).

The microbiotas of adult are assumed to be relatively stable over time which imparts resilience to disturbance, confirming continued gut function (Turnbaugh and Gordon 2009; Ley *et al*., 2006; Dethlefsen *et al*., 2008). In contrast, in the infant (full-term 2-5 years old) the chaotic shifts in the microbiome are affected with life events, such as drastic diet changes or antibiotic treatments, which result in large changes in the relative large quantity of taxonomic groups. Moreover, they found that the 2.5 years old human gut microbiome has several of the functional features of the adult microbiome (Koenig *et al*., 2010).

1.6 Potential sources of infection to neonate in NICU

Neonates characterise a unique and highly exposed patient population. Improvements in medical technology that have occurred over the last few decades have developed the survival and quality of life for neonates, particularly those infants born with extreme prematurity or with congenital defects. However, infants hospitalized in the NICU can be exposed and acquire health care-associated infections from both human and inanimate sources (Brady, 2005). Evidence supporting each of these ways is given below (Figure 1.1):

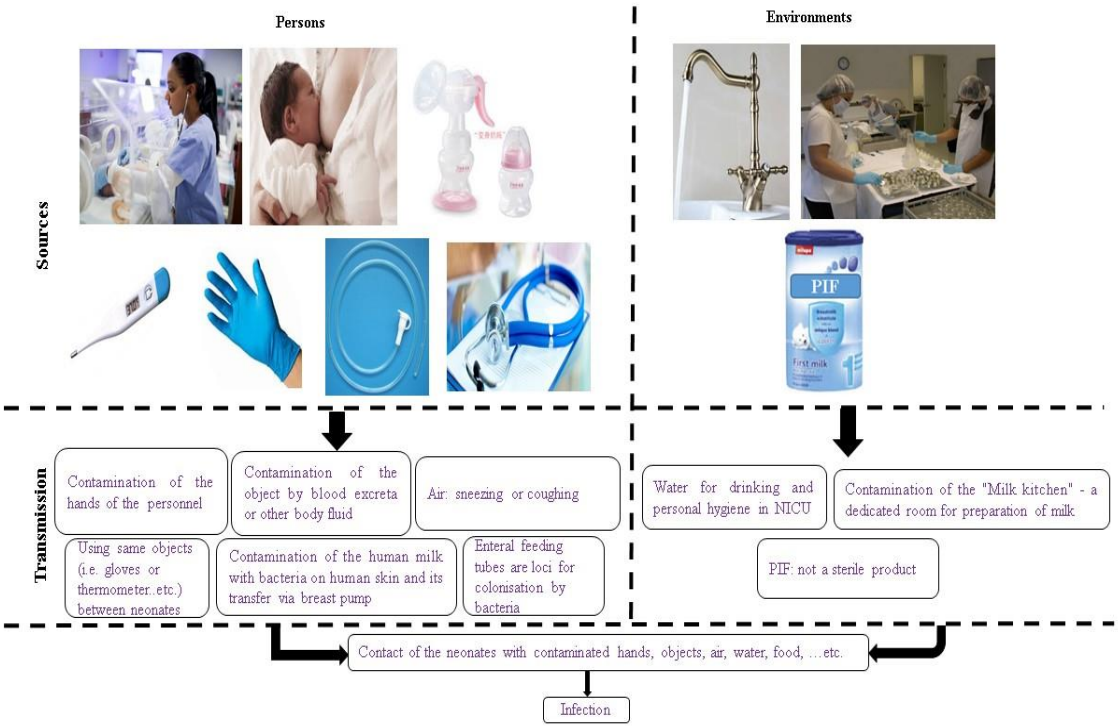


Figure 1.1: Sources of transmission of bacteria within a NICU setting.

1.6.1 Bacterial exposure of neonates human breast milk

Nutrition is necessary to the health and growth of infants and children. Breastfeeding is the best nutritional choice for infants, superior to infant formula feeding, as breast milk possesses immune factors that are effective for long term protection against infections, diseases and influences metabolism later in life (Oddy, 2001). In the NICU, most mothers are providing their breast milk to feed their premature infants (Morales and Schanler, 2007). However, breast milk has been recognized as the source of numerous bacterial infections in neonates (Qutaishat *et al*., 2003; Revathi *et al*., 1995), including *Salmonella* (Anonymous, 1978; Ryder *et al*., 1977). Several clinicians request bacterial cultures of milk from the mother for screening before allowing the milk to be fed to neonates (Ng *et al*., 2004). A previous *in-vitro* study by Lenati *et al.* (2008) found that *Cronobacter* spp. was resistant to the antimicrobial properties of breast milk, for more details see Figure 1.11.

In the last few years, there have been many studies which have demonstrated that breast milk is a prevalent source of bacteria to the infant gut and plays a role in the initiation

and growth of gut microbiota. Staphylococci, streptococci, lactococci, lactobacilli and enterococci were isolated more frequently from this biological fluid in healthy women (Heikkila *et al*., 2003; Martín *et al*., 2003).

Sometimes transmission of serious bacterial diseases to the neonate can occur through breast milk, for example, *Salmonella* species (Qutaishat *et al*., 2003), group B *streptococcus* (Kotiw *et al*., 2003), *Listeria* species (Svabic-Vlahovic *et al*., 1988), methicillin-resistant *St. aureus* (Gastelum *et al*., 2005), and *Mycobacterium tuberculosis* (Pronczuk *et al*., 2002), which is the most common species documented in cases of infection by this route, Figure 1.1

The report by Widger *et al*. (2010) states that three cases of late onset neonatal septicaemia were investigated in their unit, including one that resulted in death, which were more likely to be caused by contaminated expressed breast milk. Another report demonstrated that the contamination of breast milk has been causing fatality in neonates, but these have been related to concurrent mastitis (Gastelum *et al*., 2005; Kotiw *et al*., 2003). Recent study by Urbaniak *et al*. (2014) informed that *Enterobacteriaceae* were isolated from breast tissue of women with and without cancer in Canada and Ireland. The women were not had any symptoms of infection, however the viable of bacteria was confirmed in some samples by culture.

1.6.2 Bacterial exposure of neonates through powdered infant formula

Recently, the Food and Agriculture Organization/World Health Organization (FAO/ WHO, 2004 and 2006) have focused attention on the microbiological safety of PIF. The considerable concern for PIF was due to neonatal infections through *Cronobacter* and *Salmonella* that were associated with contaminated PIF (Caubilla-Barron *et al*., 2007; Iversen *et al*., 2008). PIF are not sterile however, they comply with the international microbiological standards (Codex Alimentarius Commission 2008). Furthermore, the report by US Centers for Disease Control and Prevention (CDC) report that in a NICU, an outbreak of *C. sakazakii* infection was associated with the use of powdered infant formula (CDC, 2002). The report also emphasised that infection through this organism often leads to a fatal disease, highlighting the significance of careful preparation, handling and use of infant formula products in the health care setting, Figure 1.1

Many types of bacteria have been isolated from PIF and the most common organisms isolated from PIF were *Clostridium perfringens*, *St. aureus*, *Bacillus cereus* and *Enterobacteriaceae* (Forsythe, 2005). *Enterobacteriaceae* including *Ent. cloacae*, *K. pneumoniae*, *K. oxytoca*, *Ent. hormaechei*, *Cit. freundii*, and *E. coli* have been isolated from PIF (Muytjens *et al*., 1988; Townsend *et al*., 2008a). These organisms have been categorised by the FAO/WHO (2004 and 2006) as category B which is 'causality plausible, but not yet demonstrated' with consideration of their potential to cause neonatal infections via the ingestion of reconstituted PIF. Though these organisms are opportunistic pathogens, there was no evidence to confirm any outbreaks in NICUs associated with their presence in contaminated PIF. The most common organisms isolated from PIF were *Cl. perfringens*, *St. aureus*, *Bacillus cereus* and *Enterobacteriaceae* (Forsythe, 2005).

There are approximately 2,000 serovars of the *Salmonella* bacterium that can cause disease in humans, and symptoms include diarrhoea, fever and vomiting which can cause serious infection in infants (Crawley and Westland, 2012). A previous study by Rodríguez-Urrego *et al*. (2010) in 2008 in Spain reported that 31 cases of *Salmonella* infection in infants occurred because of infant formula contamination, and 10 of these infants needed hospitalisation.

Powdered infant formula is made from pasteurized liquid and then spray dried. However, it is more likely that, the contamination of formula occurs from the preparer or preparation environment than from the manufacturing procedure (FAO/WHO 2004). The previous study by Weir (2002) recommended that in NICUs, the preparation of PIF should be carried out following the manufacturer's instructions by trained people under clean and aseptic conditions in a designated area. The FAO/WHO (2004) recommended that reconstitution of powdered infant formula with water >70°C, decreasing the time between reconstitution and feeding (< 2 hours), and not storing reconstituted feed at ambient temperature could reduce the risk of bacterial infection.

1.6.3 Bacterial exposure through neonatal feeding tube

In NICUs, enteral feeding tubes are commonly used to feed infants who are unable to swallow milk or formula. As per other medical inserting devices, EFT have to be clean

and sterile. Recent studies at NTU in collaboration with local NICUs revealed that organisms are present in EFT from neonates on non-formula feeding regimes (Hurrell *et al.*, 2009b). This indicates that neonate exposure to *Cronobacter* may not be exclusively through reconstituted formula, and that increased exposure to bacterial pathogens may be linked to more general feeding practices, Figure 1.1.

Hurrell *et al.* (2009a and b) studied 129 neonatal enteral feeding tubes from two local NICUs. They found that irrespective of feeding regime, neonatal enteral feeding tubes can act as loci for the bacterial attachment and multiplication of a wide range of *Enterobacteriaceae* and, hence, revealed a potentially greater risk to neonatal health than *Cronobacter* alone. The organisms frequently isolated included *E. coli*, *Ent. cancerogenus*, *Ent. hormaechei*, *K. pneumoniae*, *K. oxytoca*, *Raoutella* spp., *Se. liquefaciens* and *S. marcescens*. Additionally, *Cronobacter* spp. and *Yersinia enterocolitica* were recovered. All *S. marcescens* strains were resistant to amoxillin and co-amoxiclav.

Of additional importance was that a quarter of *Ent. hormaechei* isolates were resistant to the 3rd generation cephalosporins ceftazidime and cefotaxime. During the period of the study, *K. pneumoniae* and *S. marcescens* caused infections in the two NICUs and ESBLs were detected in two of these strains (Hurrell *et al.*, 2009b).

Hurrell *et al.* (2009b) isolated bacteria from EFT used by infants on two NICUs. The infants had received different feeding regimes: reconstituted PIF, breast milk, fortified breast milk, ready to feed formula and a mixed feeding regime. A nil by mouth group was selected as a control cohort. *Enterobacteriaceae* was isolated from 76% of the enteral feeding tubes. The highest *Enterobacteriaceae* biofilm was isolated from infants fed reconstituted PIF; an average 4.2 $\log_{10}$ cfu/tube. In fact, the PIF was reconstituted at room temperature and not with water at >70°C, as recommended by FAO/WHO. These bacteria could multiply while feeding on the formula during the time the tube was in place (48 hours) and because, unlike breast milk, PIF contains no antibacterial agents. The study found that the lowest levels of bacteria were obtained from the EFT of infants fed breast milk 52%, whereas the others ranged from 78 to 88% for mixed feeding regime and reconstituted PIF. Bacteria were obtained from the EFT of infants fed breast

milk (maximum of 5.3 $\log_{10}$cfu/tube) and infants that were nil by mouth had a maximum value of 2.7 $\log_{10}$ cfu/tube. The maximum value of breast milk was higher than the other neonates in the group and may be due to the high near neutral gastric pH (6.0). *Enterobacteriaceae* biofilms were recovered from 81% of neonates fed with sterile ready-to-feed formula. It is thought that the bacteria may have originated from the throat due to gastroesophageal reflux.

In parallel studies, Hurrell *et al.* (2009a) showed *in situ* that bacteria can grow in the nasogastric tubes to cell densities of 10^7 colony forming units/tube within 8 h, and 10^9cfu/tube within 24 hours. It is plausible that *in vivo* the biofilm will both inoculate subsequent routine feeds (every 2-3 hours) and as the biofilm ages, clumps of cells will detach and survive passage through the neonate's stomach. Subsequently, these organisms will enter the stomach as a bolus with each feed.

A major conclusion of this study was that the microbiological safety of neonatal feeds should not exclusively focus on reconstituted infant formula, and *Cronobacter* spp., but also on the general preparation and practices of enteric feeding to reduce the risk of exposure to other *Enterobacteriaceae* some of which may carry antibiotic resistance factors. Therefore, the practice of prolonged placement of EFT in neonates needs to be considered with respect to the increased risk of exposure to bacterial pathogens. Hence, biofilm formation on EFT is an important risk factor to consider with respect to neonatal infections (Hurrell *et al.,* 2009a and b). The sources of these potentially pathogenic *Enterobacteriaceae* need to be evaluated.

1.6.4 Bacterial exposure of neonates health-care workers or immediate environment

As Shown in Figure 1.1, healthcare workers and patients are the most possible sources of infectious agents and are also the most common susceptible hosts. Residents in NICU can also become infected with health care-associated infections by horizontal transmission of microorganisms spread through aerosol or contact (direct or indirect) transmission. Despite at the present time, the enhanced safety of blood products administered in hospitals, the frequent use of blood products in the stabilization of critically ill newborns allows for the potential transmission of bloodborne pathogens,

currently identified and those yet to be identified (Yeager, 1974 and Saulsbury *et al*., 1987). Likewise, Transmission of infections on contaminated medical devices is also potential and outbreaks of hospital-acquired infections have been associated to devices such as electronic thermometers, blood pressure cuffs, stethoscopes, latex gloves, masks, neckties, pens, badges and lanyards, and white coats (WHO, 2009; Uneke *et al*., 2008; Treakle *et al*., 2009).

Some studies have found that pathogens bacteria can be transmitted from out-of-hospital sources to patients through health-care workers' hands. For instance, an outbreak of postoperative *Se. marcescens* wound infections was refer to a contaminated jar of exfoliant cream in a nurse's home. An investigation suggested that the bacterial was transmitted to patients by the hands of the nurse who wore artificial fingernails (Passaro *et al*., 1997). *Se. marcescens* was transferred from contaminated soap to patients through health-care wokers'hands (Sartor *et al*., 2000).

Multidrug-resistant bacteria cause a important ratio of hospital-associated infections (Hidron *et al*., 2008; Morgan *et al*., 2010). These organisms are generally transmitted from patient-to-patient in the healthcare system via transiently contaminated healthcare workers, equipment, and the environment (Siegel *et al*., 2007). An outbreak of multidrug-resistant *Acinetobacter baumannii*, strains from patients, healthcare workers' hands, and the environment were identical (El Shafie *et al*., 2004).

Health-care waste is considered as a source of pathogenic microorganisms, that can cause contamination and give increase to infection. These microorganisms can be transmitted by direct contact, in the air, or through a diversity of vectors (Prüss, 1999).

1.7 Commonly used infant formula

The World Health Organization (WHO, 2003) and health departments across the developed and developing world recommend breastfeeding for feeding infants in the first six months of life as the best feeding method (Crawley and Westland, 2012). Infant formulas as breast milk substitutes are available for mothers who cannot or choose not to breastfeed. However, infant formulas are an artificial substitute for breast milk having inherent differences (Renfrew *et al.*, 2012) such as the exact chemical makeup of breast

milk is still unknown and cannot be manufactured. Breast milk contains mother's antibodies and numerous other defensive factors. These mature factors from the mother's immune system help the baby's immature immune system to fight against infection. In addition, infant formula requires industrial storage and delivery systems with important quality control problems. Furthermore, infant formula has no positive influence on maternal health (Crawley and Westland, 2012) whereas breast feeding leaves a life-long impact on the mother's health.

The essential components of any infant milk, irrespective of the format (powder or ready-to feed), are proteins, fats, carbohydrates, vitamins and minerals. The major infant formula manufacturers improve their own brands with a combination of each of these components. The essential nutritional properties of the majority of infant formula are very similar. Most infant formula are based on modified cows' milk (skimmed or full-fat, liquid or powder, or using whey protein concentrates) by adding lactose or other carbohydrates, vegetable and other oils, vitamins and minerals (Crawley and Westland, 2012).

Infant formula manufacturers have modified cow's milk protein (whey based) or first milks by adjusting the casein to whey ratio which is similar to breast milk (40:60). Infant formula are suitable from birth which can be either 'whey-based' or 'casein-based'. The whey-based infant formula may also have a relatively poor mineral content, specifically sodium and potassium which are required for new-born babies who have immature kidneys.

In addition, whey-based infant formula are usually the first choice if a mother does not breast-feed. While in casein-based infant formula the ratio of casein to whey is the same as cows' milk (80:20), casein-based infant formula are normally used if the baby seems not to be satisfied on a whey based product. Also, it can be helpful to delay the introduction of solids before the recommended age up to 4 to 6 months (Forsythe, 2009).

Other infant formula are based on soy protein from soya beans which is nutritionally similar to cows' milk formula. Soya infant formula is formulated for infants who cannot tolerate cows' milk because they have an allergy to the proteins in cow's milk and/or are

unable to digest the lactose in cows' milk. Usually, glucose syrups are used in soya infant formulas as a source of carbohydrates. This type of formula is used by parents who wish to feed their baby with a vegetarian product (Forsythe, 2009). The compositions of based infant formula are given in Table (1.2).

Table 1.2: The compositions of based infant formula.

Ingredients of based infant formula			
Nutrition	**Whey**	**Casein**	**Soya**
Major nutrients	Lactose Vegtable oils (contains soya lecituin) skimmed milk Demineralised water – Whey protin concentrate Galacto-oligosaccharids (GOS) Fructo-olgosaccharids (FOS) Fish oil Gluconate	Lactose Vegtable oils skimmed milk water Emulsifier (monoglycerides of fatty acids and soya lecithin) – – – – –	Glucose syrup Vegetable oils – – Emulsifler (soya lecithin) Soya protein – – – –
Vitamins	Thiamin (B1) L-carnitine Niacin (B3) Taurine Biotin Folic acid Riboflavin (B2) Vitamin A Vitamin B6 Vitamin B12 Vitamin C Vitamin D3 Vitamin E Vitamin K1 Pantothenic acid	Thiamin (B1) L-cysteine Niacin (B3) Taurine Biotin Folic acid Riboflavin (B2) Vitamin A Vitamin B6 Vitamin B12 Vitamin C Vitamin D Vitamin E Vitamin K Pantothenic acid	Thiamin (B1) L- carnitine Niacin (B3) Taurin Biotin Folic acid Riboflavin (B2) Vitamin A Vitamin B6 Vitamin B12 Vitamin C Vitamin D3 Vitamin E Vitamin K1 Pantothenic acid
Minerals	Potassium Chloride Copper Magnesium Carbonate Zinc sulphate Maganese sulphate Choline chloride Potassium iodide Sodium selenite Inositol Cytidine -5`-monophosphate Guanosine -5`-monophosphate disodium salt Uridine -5`-monophosphate disodium salt Adenosine -5`-monophosphate disodium salt Inosine -5`-monophosphate disodium salt Sodium Chloride Potassium hydroxide Calcium Citrate – Calcium hydroxide Iron lactate Calcium Phosphate Potassium Citrate Acid regulator (Citric acid)	Potassium chloride Copper sulphate Magnesium Carbonate Zinc sulphate Manganese sulphate Choline Chloride Potassium iodide Sodium selenite Inositol Cytidine- 5`-monophosphate Guanosine-5`-monophosphate disodium Uridine -5`-monophosphate disodium Adenosine -5`-monophosphate Inosine -5`-monophosphate disodium Sodium bicarbonate Potassium hydroxide Sodium citrate Ferrous sulphate – – – – –	Potassium chloride Copper sulphate Magnesium hydrogen phosphate Zinc sulphate Manganese sulphate Choline Chloride Potassium iodide Sodium selenite Inositol – – – – – – Sodium Citrate – Calcium Carbonate Iron Sulphate Calcium phosphate Potassium citrate –

1.8 Conditions and concerns in the NICU

Premature babies are those born before 37 weeks of gestation (or before the 37th week of pregnancy). They are at risk for a variety of problems such as breathing, feeding, difficulties or both, and infections. Thus, they need special medical care that only exists within NICUs or Special Care Nurseries (SCNs) (American Academy of Pediatrics, 2014). Furthermore, for premature infants, feeding by mouth is not safe and sometimes not possible because of neurological immaturity or respiratory problems. Milk in such infants is usually given through a nasogastric feeding tube. Enteral feeding is generally preferred over parenteral nutrition in the first few days of life and it helps promote endocrine adaptation and the maturation of bowel motility (McGuire *et al.*, 2004).

The WHO guidelines for hygienic preparation of PIF plan to decrease the number of bacteria in the reconstituted product by using hot water for preparation and a short time storage for any surviving organisms to grow (Codex Alimentarius Commission, 2008). However, a broader perspective is that neonates are frequently fed by using enteral feeding tubes. These tubes are commonly placed for long periods of time (more than several days) to decrease distress to the neonate by gag reflexes (Holý and Forsythe, 2014).

However, *Enterobacteriaceae* can adhere to and colonize tubes at 37°C receiving fresh feeds at regular intervals (Hurrell *et al*. 2009a). This is related to all neonates with inserted nasogastric tubes, and not only for those who are receiving reconstituted PIF. In fact, *Enterobacteriaceae* have been isolated from nasogastric tubes from neonates receiving breast milk and other feeding regimes at a rate $\geqslant 10^7$ cfu/tube (Hurrell *et al*. 2009a). Thus, regardless of the type of feed the hygienic practices and avoidance of temperature abuse are important and vital (Holý and Forsythe, 2014).

The recommendation for reconstitution of PIF with hot water (>70°C) by the FAO/WHO is not followed in all countries (e.g. USA). To avoid the contamination problems when dipping a thermometer into the reconstituted formula, the advice in the UK is to use the water that has been boiled in a kettle and left to cool for 30 minutes. With consideration of the difference in cooling curves depending on the volume of water and kind of kettle,

this is impractical for premature babies who only need small volumes of formula, and are fed at 2-hourly intervals.

The PIF includes breast milk fortifiers which are added to complement the nutritional value of breast milk. These products are not reconstituted with water and cannot be exposed to heat treatment to kill intrinsic bacteria (Holý and Forsythe, 2014).
Recently, specific UK national guidelines (under consultation) on the prevention and management of Gram-negative sepsis in neonates were developed because of a NICU outbreak in the UK (Wise, 2012).

1.9 Bacterial mechanisms of pathogenicity

Pathogenic bacteria are able to cause disease in human hosts using a number of mechanisms. Bacterial pathogens have numerous molecules that facilitate attachment to host cell targets, causing a diversity of different host responses. The molecular mechanisms through which bacteria can interact with the host can be unique to specific pathogens or conserved across several different species. The identification and characterisation of all these different strategies is the key to fighting bacterial diseases. This study focuses on mechanisms used by pathogenic bacteria to cause infectious disease.

1.9.1 The interaction of bacteria with mammalian cells

The colonisation of intestinal bacteria on the surface of intestinal walls is one of the basic ways bacteria are able to interact with mammalian cells. The intestinal walls of newborn babies are sterile, but are quickly colonised by bacteria entering the body with food and from the environment. *E. coli* is one of the first microorganisms which colonises the surface of intestinal walls of newborn babies (Grajek and Olejnik, 2004). Nevertheless, the microorganisms on the host epithelium can be affected by diverse factors, for instance, nutrition, antibiotic treatment, environmental exposure to microorganisms, and microbial colonization in the neonatal period (Lindhorst and Oscarson, 2009; Abraham and Medzhitov, 2011).

The gastrointestinal tract also has innate defence mechanisms that act against microbial survival and dissemination inside the host by washing microbes from surfaces, consisting of epithelial integrity, the rapid regeneration of old epithelial cells, fast propulsion of infected cells, autophagy, and innate immune responses (Kim *et al*., 2010). However, bacteria can invade epithelial cells and cause infections (Townsend *et al*., 2008b; Kim *et al*., 2010). Besides that, the organisms are able to form biofilms which possibly protect cells from environmental stresses and antimicrobial agents (Kim *et al*., 2008).

The preterm babies have a stratum corneum which is immature and thin as compared to that found in full term babies, children or adults. The mature stratum corneum consists of between 10 and 20 layers of cells, whereas the stratum corneum of preterm babies less than 30 weeks may have only 2 to 3 layers, which could increase the chance of invasion by harmful microbes (Evans and Rutter, 1986 and Hoeger, 2006). However, many diverse bacterial pathogens have the ability to adhere, invade, and cause damage to host cells and tissues, in addition to surviving the host defences and causing an infection (Wilson *et al*., 2002). Furthermore, several bacterial pathogens are able to secrete various components and toxins to avoid the defences of immune systems (Kim *et al*., 2010). To avoid these factors, pathogenic bacteria are also capable of creating an antiphagocytic surface layer which consists of polysaccharides or by secreting their adhesins on polymeric structures which extend out from the cell surface (Kline *et al*., 2009).

The infection of the human intestine by enteropathogenic bacteria such as *E. coli*, *Sal. enterica*, *Shigella flexneri*, and *Y. enterocolitica* is based on the capability of bacteria to adhere, invade and colonize the intestinal epithelium, survive intracellularly and be transmitted from cell to cell (Reis and Horn, 2010).Two different strategies are used by these bacteria to cause an infection in the host; firstly these bacteria are able to resist the host attack through macrophages by releasing the T3SS system proteins. Secondly they are able to survive within macrophages because of their ability to induce changes within the intracellular environment of the cell (Reis and Horn, 2010; Sansonetti, 2002).

Bacteria can interact with the host cells in two ways: attachment to the host cell without any modification to the membrane or by using the surface appendages such as microvilli (Greiffenberg *et al*., 2000). In Gram-negative bacteria, the outer membrane is an important factor which helps in the attachment of the bacteria with the external environment (Mogensen and Otzen, 2005).

1.9.1.1 Adhesion to mammalian cells

The expression of adhesive molecules on the surfaces of bacteria is an essential virulence factor. Most bacterial pathogens use adhesion to facilitate their interaction with host tissue cells. These surfaces include skin, mucous membranes, such as oral cavity, nasopharynx, urogenital tract, and deeper tissues; lymphoid tissue, gastric and intestinal epithelia, alveolar lining, endothelial tissue. The host acts to wash microbes from these surfaces by producing various mechanical forces, such as saliva secretion, coughing, sneezing, mucous flow, peristalsis, and blood flow (Wilson *et al*., 2002).

Microbial adherence factors can be produced from polypeptides (proteins) or polysaccharides (carbohydrates or sugars) which form the capsule. Protein adhesins are divided into two categories: fimbrial and afimbrial. Fimbriae (pili) are appendages that protrude from the bacterial surface and consist of proteins that are tightly packed into an array as a helical cylinder. In particular, Gram-negative bacterial pathogens depend on fimbriae for adherence such as *E. coli* (for both urinary tract infections and gastroenteritis), *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Neisseria* species (Donnenberg, 2000; Hahn, 1997).

In afimbrial adhesins made of proteins that are known as adherence factors, the contact of afimbrial adhesins with the host cell is more intimate and occur over a shorter domain than with fimbriae. Gram-negative (*Y. pseudotuberculosis*, enteropathogenic *E coli*, *Neisseria* spp.), Gram-positive (*Staphylococcus* spp. and *Streptococcus* spp.) and mycobacterial pathogens express afimbrial adhesins (Donnenberg, 2000; Merz and So, 2000; Joh *et al*., 1999; Bermudez and Sangari, 2000). The interactions between bacterial and the mucosa host such as adherence and invasion are commonly the cause of bacterial disease in the gut (Hu and Kopecko, 2008).

1.9.1.2 Invading mammalian cells

Adhesion of bacteria to host surfaces is the first step in the pathogenesis of most infections in man and animals. Some pathogens have deeper access into the host to continue the infection cycle. This pathogenic strategy can be divided into two categories: extracellular and intracellular (Wilson *et al*., 2002).

Extracellular invasion happens when a microbe breaks down the barriers of a tissue to distribute enzymes inside the host and the microbe remains outside of host cells. This is a strategy used by several pathogenic bacteria such as group A β-haemolytic *Streptococcus* and *St. aureus* which produce toxins such as haemolysin (Walker, 1998). Haemolysin are divided into three different types, α-hemolysin, β-hemolysin and γ-hemolysin. Clinical isolates, such as *E. coli* and *Serratia* spp. are able to secrete α-hemolysin (König *et al*., 1987; Welch, 1987; Schmidt *et al*., 1995). The haemolysins expressed by these species have the capability to destruct cells however, other cell types may also contribute to their spread in host tissues (Wilson *et al*., 2002).

The ability of microbes to penetrate the cells of a host tissue and survive within this situation is known as intracellular invasion. Numerous Gram-negative, Gram-positive, and mycobacterial pathogens have the capability to invade host cells (Finlay and Falkow, 1997; Cleary and Cue, 2000; Bermudez and Sangari, 2000; Dehio *et al*., 2000). *Cronobacter* strains have the ability to attach and invade Caco-2 human epithelial cells, and invade rat brain capillary endothelial cells (Townsend *et al*., 2008b).

1.9.1.3 Intracellular lifestyles

After invasion, many bacterial pathogens have the ability to survive and replicate within host cells which survive invasion such as epithelial, endothelial and macrophages. The host cells have mechanisms to damage ingested bacteria which include the creation of reactive oxidative intermediates, the decrease of pH vacuoles that contain bacteria, and the activation of destruction proteases. However, bacteria have the ability to avoid these killing mechanisms, such as survival in low acidity (Wilson *et al*., 2002). For example, Adherent-invasive *E. coli* (AIEC) isolated from Crohn's disease patients was able to replicate within macrophages in large vacuoles (Bringer *et al*., 2006). Bacteria that

survive intracellularly are possibly able to replicate and transmit between cells in the same area of infection or transfer to other areas of the body (Wilson *et al*., 2002).

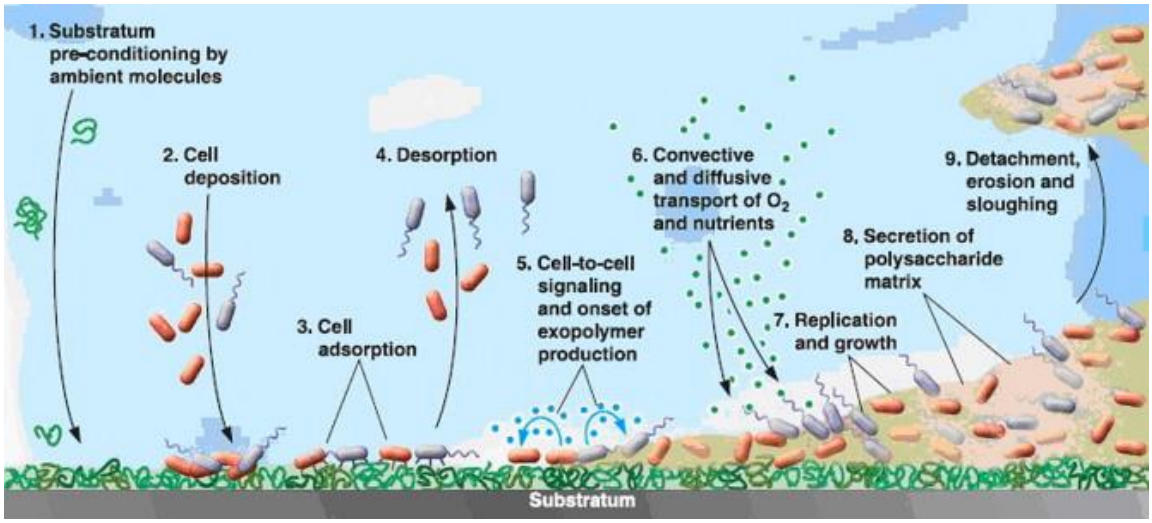
1.10 Biofilm formation

The biofilm was defined as "an assemblage of microbial cells that adhere to abiotic or biotic surfaces. Also, biofilm known as irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material" (Donlan, 2002; Davey and O'Toole 2000). In Gram-negative bacteria, several of the polysaccharides are neutral or polyanionic. The existence of ketal-linked pyruvates or uronic acids enhances their anionic properties, thus it allows association of divalent cations such as calcium and magnesium to increase the binding force in a developed biofilm (Donlan, 2002; Sutherland, 2001a).

Biofilm protect microorganisms from shear forces and low pH environments such as in the stomach. Inside the host, biofilm gives microorganisms a competitive advantage due to its protects biofilm bacteria from exposure to innate immune defences (such as opsonization and phagocytosis) and antibiotic treatments antibiotics (Scher *et al*., 2005).

The process of biofilm formation is characterized by several stages that have been identified include in Figure 1.2.

The adhesion surface is pre-conditioned by the macromolecules which are present in the bulk liquid, 2. The planktonic cells in the bulk liquid are transferred to the surface, 3. Cells are adsorbed on the surface, 4. Those cells which are adsorbed reversibly are described, 5. Some bacterial cells adsorb irreversibly on the surface, 6. Cell-cell signalling molecules are produced, 7. Transfer of substrate to and within the biofilm, 8. Cells bound to the biofilm metabolise the substrate and transfer the products out of the biofilm, 9. All of these processes occur in parallel with EPS production, replication and cell growth (Simo˜es *et al*., 2010).



Adapted from (Simo˜es *et al*., 2010).

Figure 1.2: The processes governing biofilm formation.

The attachment of bacteria will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Chae *et al*., 2006; Donlan, 2002; Millsap *et al*.,1997; Oulahal *et al*., 2008; Patel *et al*., 2007; Simo˜es *et al*., 2008). The ability of bacteria to form biofilm is related to properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and EPS production are important for biofilm formation and development (Allison, 2003; Davies *et al*., 1998; Donlan, 2002; Parsek and Greenberg, 2005; Sauer and Camper, 2001). The appendages are allowing bacterial to attach the surfaces and make the biofilm more resistant to environmental stress. The biological aspects regulating biofilm formation, which will be covered in the following sections:

1.10.1 Specialized attachment structures/surface properties of the cell

The presence of extracellular filamentous appendages and cell surface hydrophobicity may affect the rate and the extent of microbial attachment. The hydrophobicity of the cell surface is important in adhesion since hydrophobic interactions lead to a rise with an increasing non-polar nature of one or both surfaces involved, i.e., the bacterial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to another bacteria and to surfaces is based partly on the interaction of hydrophobic domains.

Numerous cells are able to produce extracellular filamentous appendages, which may play a role in the attachment process. In fact, many of such structures are known to present – flagella, pili or fimbriae, prothecae, stalks and hold-fast (Harbron and Kent, 1988).

Flagella are responsible for the motility of bacteria. Numerous types of bacteria have flagella. It is probable that the flagellum itself may form an adhesive bond with the adhesion surface (Harbron and Kent, 1988).

The primary function of flagella in biofilm formation is supposed to be in transport and in initial cell–surface interactions (Sauer and Camper, 2001). Flagella-mediated motility is assumed to overcome repulsive forces at the surface of the substratum and, as a result, a monolayer of cells forms on the adhesion surface (Daniels *et al*., 2004).

Pili or fimbriae are found on several Gram-negative bacteria including *Pseudomonas* species. They are fine, filamentous appendages ranging in width from 4–35 nm and in length up to several micrometers (Harbron and Kent, 1988). These two structures are usually straight, and are not involved in motility. Their function is only known to make cells more adhesive, because bacteria with pili can adhere strongly to other bacterial cells and inorganic particles (Harbron and Kent, 1988). According to Sauer and Camper (2001), pili and pilus-associated structures have been appeared to be significant for the adhesion to and colonization of surfaces, possibly by overcoming the initial electrostatic repulsion barrier that are present between the cell and the substratum.

The expression of fimbria is variable between pathogenic and environmental isolates. (Gerstel and Römling 2001) establish that phosphate and nitrogen depletion are signals which able to switch on the agfD promoters in the stationary phase in *Salmonella typhimurium*. Fimbria expression has been correlated with strain pathogenicity. It was discovered in 100% of *E. coli* responsible for sudden infant death syndrome, and also 55% of bovine mastitis isolates, and 38% of *E. coli* non O157 STEC (Olsén *et al*., 1989; Goldwater and Bettelheim, 2002; Cookson *et al*., 2002). Zogaj and researchers detected the production and genes responsible for cellulose and fimbria using *Citrobacter* spp. *Enterobacter* species and *Klebsiella* species. Their results also display that *Citrobacter*

species are capable to produce curli fibers but *Enterobacter* and *Klebsiella* species are unable to do. On the other hand, they discovered that genes responsible for curli fibers production were existent in *Citrobacter* species and *Enterobacter* species but not in *Klebsiella* species (Zogaj *et al*., 2003). Ten fimbrial related genes were present in all *C. sakazakii* and 38% of *C. malonaticus*, but were absent in other *Cronobacter* species (Healy *et al*., 2009).

A number of genes have been detected as requirements for the formation of curli fibers. The previous study by Zogaj *et al* (2003) has isolated *csg* genes from some species belong to *Enterobacteriacea*e such as *Citrobacter*, *Cronobacter* and *Klebsiella* spp. Furthermore, Sakellaris *et al*. (2000) isolated them from *Shigella* spp. The expression of *csg* genes is not only associated with curli expression but they are connected also with bacterial resistance to environmental factors such as desiccation. The *csg* genes were detected in strains which were unable to express curli fimbria; also not all *Salmonella* strains and *E. coli* are capable to express curli fimbria even under suitable environmental conditions (Zogaj *et al*., 2003).

The essential regulator of *csgBAC* is possibly the transcriptional regulator *csgD*. The increase of biofilm formation, invasion and bacterial virulence factors have been associated with the rise of *csgD* gene activity, being strongly controlled with genetic and environmental factors (Uhlich *et* al., 2002). It has been determined that *csgD* operon is being influenced by regulators that are grouped as *csgDEFG* and *csgBA* as divergent operons (Hammar *et al*., 1995). Gualdi and researchers (2008) found that overexpression of *csgD* can overcome temperature dependent control of curli encoding *csgBA* operon

1.10.2 Extracellular polymeric substances (EPS)

EPS are responsible to bind cells and other particulate materials together (cohesion) and to the surface (adhesion) (Allison, 2003; Sutherland, 2001b). The EPS comprises of polysaccharides, proteins, nucleic acids, lipids, phospholipids and humic substances (Jahn and Nielsen, 1998; Sutherland, 2001b; Wingender *et al*., 1999). Proteins and polysaccharides amount for 75–89% of the biofilm EPS composition, indicating that they are the predominant components (Tsuneda *et al*., 2003).

Biofilms form a gel phase around microorganisms which live inside it (Sutherland, 2001a; Wingender *et al*., 1999). The EPS matrix functions as a barrier in which diffusive transport prevails over convective transport (Sutherland, 2001b). EPS has general protective effect on biofilm forming microorganisms against adverse conditions, for example, it has frequently been shown that biofilm cells can tolerate high concentrations of biocides (Mah and O'Toole, 2001; Simo˜es and Vieira, 2009; Simo˜es *et al*., 2005).

The EPS matrix acts to delay or prevent antimicrobials from reaching target bacteria within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides (Heinzel, 1998; Mah and O'Toole, 2001). Furthermore, inside the EPS matrix the molecules necessary for cell–cell communication and community behaviour may accumulate at concentrations high enough to be effective (Pearson *et al*., 1999; Sutherland, 2001b).

Lipids and nucleic acids might have important effect on the rheological properties and thus the stability of biofilms (Neu, 1996). The extracellular DNA is required for the initial formation of biofilms by *Pseudomonas aeruginosa* and probably for biofilms formed by other bacteria that specifically release DNA (Whitchurch *et al*., 2002).

Colonic acid and cellulose are essential EPS components to form biofilm and have a similar influence on bacterial attachment to mammalian cells. Matthysse *et al.* (2008) reported that the ability of *E. coli* O157:H7 to attach to plastic surfaces as well as alfalfa sprouts and Caco-2 cell lines based on EPS. They used *E. coli* O157:H7 with deletions of *pgaC, yhjN* and *wcaD* the genes encoding for poly-ß-1,6-N-acetylglucosamine, cellulose and colonic acid respectively. Their result reported that the attachment of *E. coli* O157:H7 to alfalfa sprouts was blocked in the *pgac* mutant; the attachment was decreased 100 and 10 fold compared to the wild type strain for *yhjN* mutants and was reduced 1,000 and 100 fold for *wcaD* mutants. The attachment to Caco-2 cell line was significantly reduced for *pgaC* and *yhjN* mutants but not for *wcaD* mutant. The *E. coli* O157:H7 strains were not capable to form significant biofilm on PVC in LB agar. Mutation in the polysaccharides genes *pgaC, wacaD* and *yhjO* were capable to form third of the amount of biofilm compared to the wild type (Matthysse *et al*., 2008).

1.10.3 Cell–cell communication

Cell–cell signalling plays an important role in cell attachment and detachment from biofilms (Daniels *et al*., 2004; Donlan, 2002). Bacteria are considered to be colonial by nature and exploit elaborate systems of intercellular interactions and communications to enable their adaptation to altering environments (Davies *et al*., 1998; Fuqua and Greenberg, 2002; Sauer and Camper, 2001). Accordingly to Daniels *et al*. 2004 the successful adaptation of bacteria to changing natural conditions is based on their capability to sense and respond to the external environment and modify gene expression.

Quorum sensing is a process of cell–cell communication that based on the process of autoinduction (Eberhard *et al.,* 1981). The process of quorum sensing acts to provide a mechanism for self-organization and regulation of microbial cells (Parsek and Greenberg, 2005). It includes an environmental sensing system that allows bacteria to monitor and respond to their behaviour according to population density.

1.11 Siderophores

Bacterial pathogens require iron for survival and a diverse range of metabolic processes including amino acid synthesis, oxygen transport, respiration, nitrogen fixation, methanogenesis, the citric acid cycle and DNA biosynthesis (Sandy and Butler, 2009). The iron in human cells is very low and is bound by lactoferrin, transferrin, and ferritin (Brooks *et al*., 2004). Pathogenic bacteria synthesize and secrete small organic molecules called siderophores, which are low-molecular-weight chelators that solubilize and acquire iron from eukaryotic iron-binding proteins such as lactoferrin and transferrin (Ratledge and Dover, 2001). Thus, siderophores are considered as major virulence factors in the pathogenicity of organisms (Eijkelkamp *et al.*, 2011).

The majority of aerobic and facultative anaerobic microorganisms produce at least one siderophore (Neil, 1995). So far nearly 500 siderophores have been found from selected microorganisms and several important siderophores produced by bacteria are presented in Table 1.3 (Balagurunathan and Radhakrishnan, 2007).

Table 1.3: The important siderophores produced by bacteria.

Organisms	Siderophores
A. calcoacaticus	Acinetobactin
Ae. aerogenes	Aerobacin
Aer. hydrophila	Amonabsactin
E. coli	Enterobactin
Klebsiella spp.	Aerobactin
My. tuberculosis	Mycobactin
Salmonella	Aerobactin
St. aureus	Aureochelin
P. aeruginosa	Pyoverdin and pyochelin
V. cholerae	Vibriobactin
Y. pestis	Yersniabactin

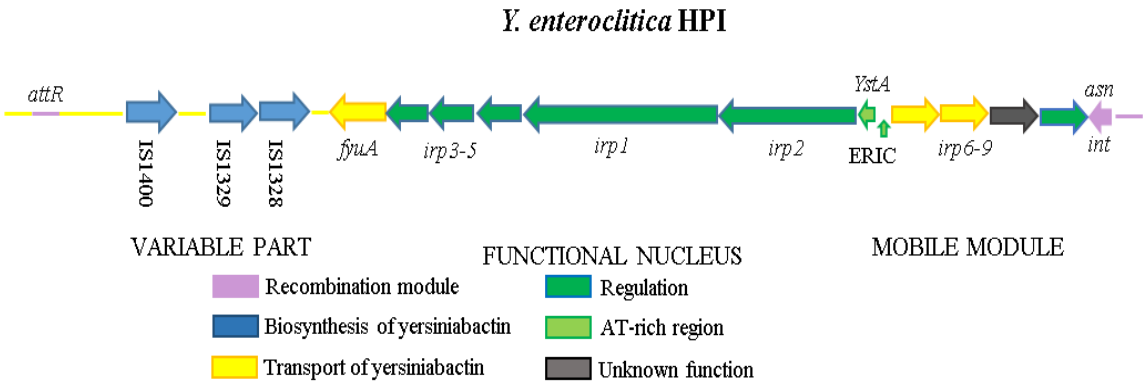
Yersiniabactin is part of an iron-uptake system discovered in several species of the family *Enterobacteriaceae* (Bach *et al*., 2000; Schubert *et al*., 2000) and was first described in *Yersinia* spp., where the genes are located on a high-pathogenicity island (HPI) (Carniel, 2002). Several previous studies have reported that *Yersinia* spp., *E. coli* and possible *K. pneumoniae* strains having the HPI are more virulent than strains lacking this island (Bearden *et al*., 1997; Carniel *et al*., 1992; Lin *et al*., 2008).

There are many studies that have demonstrated that pathogenic strains of *Yersinia*; *Y. pestis* (Fetherston *et al*., 1992), *Y. pseudotuberculosis* (Buchrieser *et al*.,1998) and *Y. enterocolitica* (Carniel *et al*., 1996) carry a pathogenicity island known as the HPI (Carniel *et al*., 1996) for the reason that its existence is essential for the expression of a high-virulence phenotype. The HPI of *Y. pestis* is known as the *pgm* locus, which consists of about 102 kb of chromosomal DNA and comprises the *irp1* and *irp2* and *fyuA* or *psn* genes included in iron storage and uptake the hemein storage (hms) locus (Perry *et al*., 1990), see Figure 1.3 .

The *irp1* and *irp2* genes code for the two iron-repressible high-molecular-weight proteins HMWP1 and HMWP2, which apparently are involved in the production of yersiniabactin (Lucier *et al*., 1996); and the *fyuA* or *psn* gene plays a significant role for ferric yersiniabactin uptake or pesticin sensitivity as it serves as coding for the yersiniabactin receptor. *FyuA* also acts as a receptor for pesticin (Carniel *et al*., 1992;

Carniel *et al*., 1989; Heesemann *et al*., 1993; Rakin *et al*., 1994). HMWP1 and HMWP2 are found only in pathogenic strains of *Yersinia* and are associated with virulence (Carniel *et al*., 1987 and 1989).

In the Netherlands, a nationwide outbreak was caused by multidrug resistance *Ent. hormaechei* outbreaks (EHOS). These organisms appeared to possess mechanisms to acquire iron from their environment by producing yersniabactin which were found on the HPI. The HPI was transferred to other *Enterobacter* spp. *in vivo* which possibly played a role in the epidemic behaviour (EHOS) (Paauw, 2008b).



Adapted from (Tang *et al*., 2014)

Figure 1.3: Genetic and structure organization of the high pathogenicity island of *Y. enterocolitica*.

1.12 Diversity of antibiotics resistance in *Enterobacteriaceae*

Over the past several decades, the widespread use of antibiotics has led to the appearance of antibiotic resistant strains of many bacteria which then become a serious clinical and public health problem (Wilson *et al*., 2002). Antibiotic resistance among nosocomial pathogens is a cause of concern (Wood *et al*., 1996; Goldmann *et al*., 1996). Gram-negative and Gram-positive bacteria have acquired resistance to many types of antimicrobial drugs (Wilson *et al*., 2002). Many antibiotic resistant bacterial strains have

acquired multidrug resistance, which represents a cause for significant concern, including diarrhoeal pathogens, for example, *Shigella*, *Salmonella*, *E. coli*, and *Enterococcus faecium*; respiratory pathogens such as *K. pneumoniae* and *P. aeruginosa*; urinary tract pathogens like *E. coli*, and *M. tuberculosis*, which remains the leading cause of death from a single infectious disease worldwide (Mazel and Davies, 1999; Wood *et al*., 1996).

Enterobacteriaceae with *E. coli* are by far the most important pathogens for humans and have been known as the most common source of community and hospital-acquired infections. They have the facility to spread easily among humans by hand carriage and contaminated food and water and have the ability to acquire genetic material by horizontal gene transfer, mediated mostly by plasmids and transposons (Partridge, 2011; Stokes and Gillings, 2011; Toleman and Walsh, 2011). For these reasons, the emerging multidrug resistance in *Enterobacteriaceae* is of most importance for clinical therapy.

The β-lactams are different antibiotic molecules that are composed of four groups: penicillins, cephalosporins, monobactam, and carbapenems, according to their chemical structures. The latest developed molecules that have the broadest spectrum of activity are carbapenems (imipenem, ertapenem, meropenem, and doripenem) (Nordmann *et al*., 2012). Multidrug-resistant *Enterobacteriaceae* produce ESBLs which are the most important contributing factor to β-lactam resistance (Livermore, 2003). β-lactamases are enzymes produced by bacteria that act to inactivate β-lactam antibiotics by hydrolysis. These enzymes are divided into four groups based on their activity profile as shown in Table 1.4 (Nordmann *et al*., 2012).

Table 1.4: Diversity of β-lactam resistance in *Enterobacteriaceae*.

enzyme of β-lactamases	β-lactam antibiotics	
	inactivate	no affect
Penicillinases	penicillins	cephalosporins, aztreonam, or carbapenems
Cephalosporinases	cephalosporins and aminopenicillins	other penicillins (carboxy- and ureido-penicillins), aztreonam, and carbapenems
Extended-spectrum β-lactamases (ESβLs)	all β-lactams	carbapenems
Carbapenemases	carbapenems and may also deactivate other types of β-lactam molecules, depending on the enzyme	-

The ESBLs are β-lactamases which have the capability to hydrolyse and cause resistance to different types of the β-lactam antibiotics, such as the expanded-spectrum (or third-generation) cephalosporins and monobactams, but not the cephamycins and carbapenems (Bradford, 2001). Bacteria that are able to produce ESBLs are still an important reason for therapy failure with cephalosporins and have serious effects on infection control (Paterson and Bonomo, 2005).

The majority of ESBLs have been divided into three groups: TEM, SHV, and CTX-M types (Paterson and Bonomo, 2005). Members of the family *Enterobacteriaceae* are most commonly express plasmid-encoded β-lactamases such as TEM-1, TEM-2, and SHV-1 that confer resistance to penicillins but not to expanded-spectrum cephalosporins (Leverstein-van Hall *et al*., 2002). The majority of ESBL-producing organisms isolated worldwide are *K. pneumoniae* and *E. coli*, but these enzymes have also been discovered in many other members of the *Enterobacteriaceae* family and, in particular, non-fermentors (Jacoby and Munoz-Price, 2005).

The Infectious Diseases Society of America reported that ESBL-producing *Klebsiella* spp. and *E.coli* are together one of the six drug-resistant microbes against which new therapies are urgently needed (Talbot *et al*., 2006). ESBLs have continued to increase in diversity and spread and are now a global health concern (Serefhanoglu *et al*., 2009; Martinez *et al*., 2012; Lautenbach *et al*., 2001). A recent trend is the emergence of

community-onset bloodstream infections caused by ESBL-producing bacteria, particularly CTX-M producing *E. coli*. These infections are now rare, but it is possible that, in the near future possible the clinicians will be regularly confronted with hospital varieties of bacteria causing infections in patients from the community (Thenmozhi *et al*., 2014).

Mostly phenotypic methods are used in clinical diagnostic laboratories because these tests are easy to do, are cost effective, and have been included in most automated sensitivity systems, making them widely accessible (Wiegand *et al*., 2007). However, phenotypic techniques are not able to differentiate between the specific enzymes responsible for ESBL production types (SHV, TEM, and CTX-M). Numerous research or reference laboratories are using genotypic methods to identify the specific gene responsible for the production of the ESBL (Woodford and Sundsfjord, 2005).

Since 2000, many surveys from different European countries, including Spain (Rodríguez-Baño *et al*., 2004), Italy (Mugnaioli *et al*., 2006), Greece (Pournaras *et al*., 2004), the UK (Woodford *et al*., 2004) and Canada (Pitout *et al*., 2007) have reported an alarming increase in resistance of bacteria to other classes of antimicrobial agents between ESBL-producing bacteria isolated from community sites. These surveys exhibited co-resistance to co-trimoxazole, tetracycline, gentamicin, and ciprofloxacin. For example, in Canada more than 66% of isolates were resistant to ciprofloxacin (Pitout *et al*., 2007). These studies also presented that strains producing CTX-M enzymes were significantly more resistant to ciprofloxacin than strains missing polymerase chain reaction (PCR) evidence for *bla*CTX-M genes (Pitout *et al*., 2007; Pitout *et al*., 2004).

Hurrell *et al*. (2009b) determined the antibiograms for the *Enterobacteriaceae* strains isolated from EFT in NICU and they found that all the *S. marcescens* strains were resistant to amoxillin and co-amoxiclav. Also, a quarter of *Ent. hormaechei* isolates were resistant to the 3rd generation cephalosporins, ceftazidime and cefotaxime. During the study period *K. pneumoniae* and *S. marcescens* caused infections in the two NICUs. In addition, ESBLs were discovered in three of these strains (Hurrell *et al.*, 2009b).

The infant intestinal flora is influenced by the feeding regime. *Enterobacteriaceae* are present in neonatal nasogastric enteral feeding tubes in NICU, but there is no study determined the risk of these strains. This study focuses on assessing the risk to neonates from opportunistic bacterial pathogens (*Enterobacteriaceae*) which have been isolated from two sources; human mastic breast milk and also neonatal nasogastric enteral feeding tubes. The overall purpose of the current study was to evaluate the risk to neonates posed by ingestion of specified *Enterobacteriaceae* either from contaminated milk or from infant formula.

1.13 Objectives

The initial studies used PFGE to genetically fingerprint the strains isolated from the NICUs by other researchers. This was determined by the prevalence and persistence of particular *Enterobacteriaceae* species, for example, whether certain strains persist in the NICUs, and verified whether they colonise the tubes of unrelated neonates over prolonged periods of time. The second part of the project was to focus on the virulence potential of *K. oxytoca, Ent. hormaechei* and *Ent. ludwigii*.

To extend the earlier studies by Hurrell *et al.* (2009a and b) of *Enterobacteriaceae* from EFT, additional strains from mastic breast milk (MBM) were included. These were obtained from a study by Delgado *et al.* (2008). *Klebsiella* strains have been selected as they are both a common cause of neonatal infections and were frequently isolated from the EFT. An additional reason is that when Delgado *et al*. (2008) examined the bacterial diversity of MBM, they isolated a number of *Klebsiella* strains, and these have been made available to us for comparative studies. Despite common expectation, breast milk is not sterile and can contain a range of bacteria.

There was additional merit for a study to include *Klebsiella* as it has been isolated from similar sources from two separate countries (UK and Spain) and this has considerable comparative value. The second organism of interest, *Ent. hormaechei*, was isolated from both the previous Nottingham NICU studies of Hurrell *et al.* (2009b). This organism had come to our attention through a series of semi-related studies, as follows:

1. Dr Stacy Townsend (former NTU post-doctoral researcher) acquired 'Cronobacter' strains from a hospital for further analysis. Further studies using PFGE revealed that they were clonal, and were from a NICU outbreak which the hospital was not aware of. Subsequent 16S rDNA sequencing reidentified the strains as *Ent. hormaechei* (Townsend *et al.,* 2008a). Hence, the hospital had not only not recognised an outbreak but had also misidentified the organisms.

2. NTU acquired a quinolone resistant 'Cronobacter' from Poirel *et al*. (2007) as it is an unusual trait for the organism. However, 16S rDNA sequencing showed the strain was *Ent. hormaechei* and this indicates the difficulty in treatment of infections due to this organism as well as its identification in the clinical setting.

3. Muytjens *et al*. (1988) highlighted the incidence of *Cronobacter* spp. in powdered infant formula. This paper has been highly cited to date as a key primary study on the topic. However, the strains were identified in 1988 using biochemical profiling, whereas NTU used 16S rDNA sequence analysis to reidentify the strains, some of which were *Ent. hormaechei* (Townsend *et al*., 2008a). Hence, this indicates powdered formula as a source of neonate exposure.

4. *Enterobacteriaceae* isolates from EFT from QMC and NCH hospitals included *Ent. hormaechei* (Hurrell *et al.,* 2009b). Many of these strains were resistant to the 3rd generation cephalosporins, ceftazidime and cefotaxime, and ESBLs were detected. This demonstrates that neonates are directly exposed to antibiotic resistant strains in NICUs during feeding.

5. Comparative bacterial death rates of *Enterobacteriaceae* revealed that the *Ent. hormaechei* strain isolated from neonatal enteral feeding tube (4 above) was more heat resistant than *Sal.* Seftenberg. This later organism is normally recognised as the most heat resistant *Enterobacteriaceae* and heat treatment processes in industry use this later organism to ensure their processes are efficient (Forsythe; FSA report 2009). This shows that the organism has a greater chance of surviving during the reconstitution of infant formula than other organisms, and that procedures may need to be modified to take this into account.

In addition to *Ent. ludwigii* obtained from a study by Delgado *et al*. (2008), we also received *Ent. ludwigii* strain (NTU1439) from Nottingham QMC hospital that was isolated from a case of neonatal meningitis. This organism was included in the project as it reinforced the importance of evaluating the risk of neonatal infection by *Ent. ludwigii*.

Ent. aerogenes and *Ent. cloacae* were not included in this study because, at the time this study was initiated, these strains were considered as *K. oxytoca*. However, later we identified these strains as *Ent. aerogenes* and *Ent. cloacae* using 16S rDNA sequence analysis.

This study compares the physiological and virulence traits between bacterial species which neonates are exposed to from MBM and EFT. Only a limited comparison was made according to the source of strains. However, this was not possible for *Ent. ludwigii* and *Ent. hormaechei* as these were not isolated from MBM and EFT respectively.

Finally, *K. pneumoniae* strains obtained from Jordan which have been isolated from EFT were also analysed, but only for PFGE profile due to time constraints.

1.14 Project Aims

In particular, the specific aims are:

1. Characterise *Ent. ludwigii*, *Ent. hormaechei* and *Klebsiella* spp. strains according to standard phenotyping methods and compare with genotyping.

2. Determine physiological attributes of the strains with respect to heat tolerance, biofilm formation, cell capsule production and acidic pH survival (pH 3.5).

3. Determine the virulence potential of strains isolated from MBM and EFT using tissue culture technique and by identifying the presence of virulence factors.

Chapter 2:MATERIALS AND METHODS

2.1 GENERAL MATERIALS

2.1.1 Bacterial strains

A total of 228 strains of *Enterobacteriaceae* from different sources were used in this study. One hundred thirty-three strains had been isolated from neonatal EFT from two local Nottingham hospitals (QMC and NCH), (Table 2.1- 2.3) and reported in a previous Nottingham NICU study by Hurrell *et al.* (2009b). Twenty strains had been isolated from MBM by the lactation consultants at different primary health-care centres in Spain (Delgado *et al.,* 2008) (Table 2.4). Seventy five EFT and flushed milk isolates of *K. pneumoniae* from Jordan were also analysed, but only for PFGE profile due to time constraints (Table 2.5 part 1 and 2).

The following studies were carried out on the three sets of strains:

- Initial experiments used strains from two different sources, EFT and MBM, to determine their relatedness by using PFGE technique (Tables 2.1-2.4). PFGE subtyping of EFT isolates from QMC and NCH was by Alkeskas in our research group as part of his PhD project (Table 2.1- 2.3).
- Further experiments used strains from MBM (Table 2.4) and EFT isolates from QMC and NCH (Table 2.6). These strains were used to determine their physiological traits, such as heat tolerance, biofilm formation, capsule formation and acid tolerance at pH 3.5. In addition, these organisms were used to investigate their ability to attach and invade Caco-2 cell line.
- Twenty three strains were chosen for virulence factors determination, as shown in (Table 2.7). This part of the project focused on the virulence potential of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cloacae*, *Ent. aerogenes* and *K. oxytoca* isolated from two different sources.
- *Ent. hormaechei*, and *Ent. ludwigii* strains from the MBM and EFT isolates (QMC and NCH) collection were used for determining attachment and invasion HBMEC and rBCEC4 cell lines (Table 2.8).

Table 2.1: Description of *Ent. hormaechei* and *Ent. aerogenes* strains isolated from enteral feeding tubes at neonatal intensive care units in Nottingham.

Species	Strain number	Date of isolation	Source	Neonate
Ent. cloacae	779	16/01/2007	NCH	2
Ent.hormaechei	790			4
Ent. cloacae	789	23/01/2007		12
"	795	30/01/2007		17
"	797			17
"	798			18
"	799			18
"	800			19
"	801			20
"	802			21
"	803			22
"	979	16/02/2007		36
"	980			36
"	981			36
"	983			36
"	986			36
"	987			36
"	993			36
"	997			36
"	856	17/04/2007		50
"	859			51
"	860			52
"	861			52
"	862			52
"	863			52
"	960	23/05/2007		83
Ent. cloacae	1028	16/10/2007	QMC	108
Ent.hormaechei	1044			115
"	1052			117
"	1033			110
"	1034			110
"	1035			110
"	1074			117
"	1032			110
"	1053			117
"	1075			117
"	1027			108
Ent. aerogenes	1058			118
"	1056	22/10/2007		119
Ent.hormaechei	1038			113
"	1039			113
"	1040			113
"	1066	12/11/2007		126
"	1067			126
"	1081			126
"	1084			126
"	1068	19/11/2007		128
"	1069			128
"	1089			128

NCH indicates Nottingham City Hospital and QMC indicates Queen's Medical Centre.

Table 2.2: Description of *Ent. cancerogenus* strains isolated from enteral feeding tubes at neonatal intensive care units in Nottingham.

Species	Strain number	Date of isolation	Source	Neonate
Ent. cancerogenus	782	16/01/2007	NCH	7
"	783			8
"	781	17/01/2007		6
"	784	23/01/2007		9
"	806	06/02/2007		24
"	807			24
"	808			24
"	810			26
"	814	11/02/2007		27
"	815			27
"	816			27
"	817			27
"	818	13/02/2007		28
"	824	16/02/2007		36
"	982			36
"	985			36
"	988			36
"	989			36
"	992			36
"	994			36
"	996			36
"	998			36
"	990	17/02/2007		37
"	991			37
"	825			37
"	826			37
"	827	20/02/2007		33
"	828			33
"	829			33
"	831			33
"	834			35
"	957	22/05/2007		81
"	966			86
"	969			87
"	972			87
"	842	20/03/2007		42
"	845	02/04/2007		45
"	847			45
"	848	10/04/2007		48
"	850			48
"	851			48
"	907	24/04/2007		58
"	908			58
"	909			58
"	1059	16/10/2007	QMC	119
"	1071			108
"	1037			112
"	1042	22/10/2007		128
"	1054			118
"	1077	31/10/2007		120
"	1088	19/11/2007		120

NCH indicates Nottingham City Hospital and QMC indicates Queen's Medical Centre.

Table 2.3: Description of *Klebsiella* spp. strains isolated from enteral feeding tubes at neonatal intensive care units in Nottingham.

Species	Strain number	Date of isolation	Source	Neonate
K. pneumoniae	778	23/01/2007	NCH	1
"	785			9
"	787			10
"	788			11
"	792			13
"	794			14
K. oxytoca	832	16/02/2007		36
"	833			36
"	830	20/02/2007		33
K. pneumoniae	839	28/02/2007		39
"	841			39
"	976	30/05/2007		89
"	977			89
"	999	05/06/2007		94
"	1004			94
"	1006			94
"	1000			95
"	1002			95
"	1005			95
"	1001			96
"	1003			96
"	1012	12/06/2007		103
"	1013			103
"	1014			103
K. pneumoniae	1046	22/10/2007	QMC	116
"	1048			116
"	1049			116
K. oxytoca	1078	31/10/2007		120
"	1079			121
K. pneumoniae	1090	19/11/2007		129
"	1091			129
"	1092			129
"	1093			129

NCH indicates Nottingham City Hospital and QMC indicates Queen's Medical Centre.

Table 2.4: Description of strains isolated from Spain from MBM. All strains in this table were used for the characterisation and identification of bacterial isolates; chapter 3 and determining physiological traits; chapter 4.

Species	Strain	Location	Source of isolation
Ent. ludwigii	1349	HCM	MBM
"	1351	"	"
"	1352	"	"
"	1348	"	"
"	1366	"	"
Ent. cancerogenus	1350	HCM	MBM
"	1357	"	"
"	1358	"	"
"	1359	"	"
"	1360	"	"
"	1361	"	"
"	1362	"	"
"	1363	"	"
"	1364	"	"
"	1365	"	"
"	1367	"	"
"	1355	"	"
K. oxytoca	1353	HCM	MBM
"	1354	"	"
"	1356	"	"

HCM indicates Hospital Clinico of Madrid and MBM indicates mastic human breast milk.

Table 2.5: Description of *K. pneumoniae* strains isolated from neonatal EFT used in Jordan, part 1.

Species	Strains	Location	Source of isolates	Feeding regime	Age of infant	Neonatal Number	Date of isolation
K. pneumoniae	1681	PRH P	Flushed milk	bebelac	21 days old	1	23/07/2011
"	1682	"	"				
"	1683	PRH J	Tube		9 days old	2	
"	1684	"	"				
"	1685	"	"				
"	1713	"	Flushed milk				
"	1714	"	"				
"	1686	PRH A	"		21 days old	3	04/07//2011
"	1687	"	"				
"	1688	"	Tube				
"	1689	"	"				
"	1690	PRH B	"		12 days old	4	
"	1691	"	"				
"	1692	"	Flushed milk				
"	1693	PRH C	Tube		37 days old	5	09/07/2011
"	1694	"	"				
"	1695	"	Flushed milk				
"	1696	"	"				
"	1697	PRH D	Tube		23 days old	6	
"	1698	"	"				
"	1699	KAH 17	Flushed milk	neosure	26 days old	7	04/07//2011
"	1700	"	"				
"	1701	KAH16	Tube		24 days old	8	31/05/2011
"	1702	"	"				
"	1703	"	Flushed milk				
"	1704	"	"				
"	1705	KAH1 15	Tube	S26	15 days old	9	
"	1706	"	"				
"	1707	"	Flushed milk				
"	1708	"	"				
"	1709	PRH H	Tube		16 days old	10	06/07/2011
"	1710	"	"				
"	1711	"	Flushed milk	bebelac			
"	1712	"	"				
"	1715	PRH L	"		9 days old	11	23/07/2011
"	1716	"	"				
"	1717	PRH F	Tube		35 days old	12	07/07/2011

PRH indicates Princesses Rahma Hospital, KAH indicates King Abdulla Hospital and the letters or numbers after the abbreviations (KAH or PRH) are indicating code of infant information. Flushed milk indicates lumen liquid from enteral feeding tube.

Table 2.5: Description of of *K. pneumoniae* strains isolated from neonatal EFT used in Jordan, part 2.

Species	Strains	Location	Source of isolates	Feeding regime	Age of infant	Neonatal No.	Date of isolation
K. pneumoniae	1718	PRH O	Tube	bebelac	9 days old	12	23/07/2011
"	1719	"	"			13	
"	1720	"	Flushed milk				
"	1721	"	"				
"	1722	PRH I	"		39 days old	14	06/07/2011
"	1723	"	"				
"	1724	"	Tube				
"	1725	PRH G	Flushed milk		15 days old	15	
"	1726	"	"				
"	1727	KAH 6	Tube		5 days old	16	22/05/2011
"	1728	"	"				
	1729	PRH N	Flushed milk		14 days old	17	15/07/2011
"	1730	PRH E	Tube		19 days old	18	09/07/2011
"	1731	"	"				
"	1732	"	Flushed milk				
"	1733	"	"				
"	1734	KAH 34	Tube	neosure	18 days old	19	07/12/2011
"	1735	"	"				
"	1736	"	Flushed milk				
"	1738	PRH T	"	S26	15 days old	20	10/12/2011
"	1737	PRH S	tube		36 days old	21	
"	1739	"	Flushed milk				
"	1740	"	"				
"	1741	PRH Q	"		8 days old	22	
"	1742	"	Tube				
"	1743	"	"				
"	1744	PRH AD	Tube		13 days old	23	15/12/2011
"	1745	"	Flushed milk				
"	1746	PRH AE	"		7 days old	24	
"	1747	"	"				
"	1748	PRH AT	Tube			25	
"	1749	"	"				
"	1750	PRH AI	Flushed milk		24 days old	26	
"	1751	"	Tube				
"	1752	PRH Y	"		10 days old	27	12/12/2011
"	1753	"	"				
"	1754	PRH W	Flushed milk		11 days old	28	20/12/2011
"	1756	"	"				

PRH indicates Princesses Rahma Hospital, KAH indicates King Abdulla Hospital and the letters or numbers after the abbreviations (KAH or PRH) are indicating code of infant information. Flushed milk indicates lumen liquid from enteral feeding tube.

Table 2.6: Summary table of selected *Ent. hormaechei*, *Ent. cloacae*, *Ent. aerogenes*, *Ent. cancerogenus* and *K. oxytoca* strains from EFT isolates (QMC and NCH) collection, used for determining physiological traits; chapter 4.

Species	Strain	Location	Source of isolates	Neonate Number	Date of isolation	Age of infant	Feeding regime
Ent. hormaechei	790	NCH[a]	EFT	4	16/01/2007	> 4weeks	breast milk
"	795	"	"	17	30/01/2007		breast milk & powder infant formula
"	798	"	"	18		2-3weeks	nil by mouth
"	860	"	"	52	17/04/2007		breast milk with fortifier
"	1034	QMC[b]	"	110	16/10/2007	1-2 weeks	breast milk & pre-made formula
"	1038	"	"	113	22/10/2007	> 4 weeks	pre-made formula
"	1053	"	"	117	16/10/2007	2-3 weeks	breast milk & pre-made formula
"	1075	"	"	117			
Ent. cloacae	779	NCH[a]	EFT	1	16/01/2007	> 4 weeks	pre-made formula
"	789	"	"	12	23/01/2007		breast milk with fortifier
Ent. aerogenes	1056	QMC[b]	"	118	22/10/2007	< one week	breast milk
Ent. cancerogenus	806	NCH[a]	EFT	24	06/02/2007	> 4 weeks	breast milk with fortifier
"	824	"	"	36	16/02/2007	3-4 weeks	breast milk
"	845	"	"	45	02/04/2007	2-3 weeks	pre-made formula
"	848	"	"	48	10/04/2007	> 4 weeks	infant formula
"	909	"	"	58	24/04/2007	2-3 weeks	pre-made formula
"	957	"	"	81	22/05/2007	3-4 weeks	breast milk with fortifier
"	1037	QMC[b]	"	112	16/10/2007	1-2 weeks	
"	1042	"	"	128	22/10/2007	> 4 weeks	breast milk with fortifier
"	1077	"	"	120	31/10/2007	1-2 weeks	
K. oxytoca	832	QMC[b]	EFT	36	16/02/2007	3-4 weeks	breast milk
"	1078	"	"	120	31/10/2007	1-2 weeks	breast milk with fortifier
"	1079	"	"	121		< one week	pre-made formula

(a) NCH Nottingham City Hospital, (b) QMC Queen's Medical Centre.

Table 2.7: Summary table of selected *Ent. hormaechei, Ent. cloacae, Ent. aerogenes, Ent. ludwigii* and *K. oxytoca* strains from the MBM and EFT isolates (QMC and NCH) collection, used for determining virulence factors; chapter 5.

Species	Strain	Source of isolates	Source
Ent. hormaechei	790	EFT	NCH[a]
"	795	"	"
"	798	"	"
"	860	"	"
"	1034	"	QMC[b]
"	1038	"	"
"	1053	"	"
"	1075	"	"
Ent. cloacae	779	EFT	NCH[a]
"	789	"	"
Ent. aerogenes	1056	EFT	QMC[b]
Ent. ludwigii	1349	MBM	HCM[c]
"	1351	"	"
"	1352	"	"
"	1348	"	"
"	1366	"	"
"	1439	CSF	QMC[b]
K. oxytoca	832	EFT	QMC[b]
"	1078	"	"
"	1079	"	"
"	1353	MBM	HCM[c]
"	1354	"	"
"	1356	"	"

(a) NCH Nottingham City Hospital, (b) QMC Queen's Medical Centre, (c) HCM Hospital Clinico of Madrid. EFT: Indicates Enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates cerebrospinal fluid. Pulsotype for MBM and CSF strains is detailed in this study (chapter 3) whereas PFGE pulsetype for EFT isolates were by Alkeskas in our research group as part of his project.

Table 2.8: Table of selected *Ent. hormaechei*, and *Ent. ludwigii* strains from the MBM and EFT isolates (QMC and NCH) collection, used for determining attachment and invasion HBMEC and rBCEC4 cell lines; chapter 5.

Species	Strain	Source of isolates	Location
Ent. hormaechei	790	EFT	NCH[a]
"	795	"	"
"	798	"	"
"	860	"	"
"	1034	"	QMC[b]
"	1038	"	"
"	1053	"	"
"	1075	"	"
Ent. ludwigii	1349	MBM	HCM[c]
"	1351	"	"
"	1352	"	"
"	1348	"	"
"	1366	"	"
"	1439	CSF	QMC[b]

(a) NCH Nottingham City Hospital, (b)QMC Queen's Medical Centre, (c) HCM Hospital Clinico of Madrid. EFT: Indicates Enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates Cerebrospinal fluid. Pulsotype for MBM and CSF strains is details in this study (chapter 3) whereas PFGE pulsetype of EFT isolates were by Alkeskas in our research group as part of his project.

2.1.2 Bacterial cultivation

2.1.2.1 Tryptone soya agar (TSA)

Tryptone soya agar (CM131B, Oxoid Thermo Fisher Scientific) was prepared according to the manufacturer's instructions. Briefly, 40g of Tryptone soya agar 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 4ºC until required.

2.1.2.2 Tryptone soya broth (TSB)

Tryptone soya broth (CM0989B, Oxoid Thermo Fisher Scientific) was prepared by dissolving 30g of TSB in 1 litre of distilled water, and autoclaving at 121°C for 15 minutes. The media was stored in a cool and dark place until required.

2.1.2.3 Milk agar

To prepare milk agar media, 3g of agar (LP001, Oxoid Thermo Fisher Scientific) and 0.4g of ammonium sulphate were dissolved in 40ml of distilled water, and autoclaved at 121ºC for 15 minutes. 200ml of milk based ready to use formula whey (Cow and Gate First Infant Milk), casein (SMA Extra Hungry Ready to use) and soy-based (Cow and Gate infasoy) infant formula was warmed at 55ºC and mixed with the autoclaved agar. The medium was dispensed into Petri dishes, and stored in a cooled place until required.

2.1.2.4 Saline distilled water

One saline tablet (BR0053G, Oxoid Thermo Fisher Scientific) was dissolved in 500ml distilled water to prepare 0.85% saline that was autoclaved at 121°C for 15 minutes.

2.1.2.5 Luria-Bertani broth (LB)

Luria-Bertani Broth (L3022, Sigma Aldrich; USA) was prepared by dissolving 25g of Luria-Bertani in 1 litre distilled water. It was dispensed in the required volumes and autoclaved at 121ºC for 15 minutes. The media was stored in a cool and dark place until required.

2.1.2.6 Iso-Sensitest agar (ISO)

Distilled water (1 litre) was mixed with 31.4g ISO agar (CM0471, Oxoid Thermo Fisher Scientific), brought to boil to dissolve the medium completely and then sterilised by autoclaving at 121°C for 15 minutes. The plates were stored in a cool place until required.

2.1.2.7 Long term storage of strains

In a 2ml Eppendorf tube 800µl of TSB broth was mixed with one colony from the TSA plate and 200µl of 80 % glycerol was added and stored in a -80°C freezer.

2.1.3 Mammalian cell lines

In this study, four mammalian cell lines (Caco-2, HMBEC, rBCEC4 and U937) were used to determine *Enterobacteriaceae* virulence towards host cells. The cell lines were stored in liquid nitrogen, with the date, cell line type and passage numbers clearly labelled.

2.1.3.1 Human colonic carcinoma epithelial (Caco-2) cell line

Human colonic carcinoma epithelial cell line (Caco-2) was received from the European Collection of Cell Cultures (ECACC# 86010202). The cell line was originally from a human colon adenocarcinoma. The cell lines are able to form a well-differentiated cell monolayer and are similar to small intestinal enterocytes. Therefore, it is widely utilized in studies of pathogen-host cell interaction (Szymanski *et al*., 1995). Cells were used between passages seven and twelve.

2.1.3.2 Human brain microvascular endothelial cells (HBMEC)

The human brain microvascular endothelial HBMEC cell line was obtained from Dr M. Rittig (University of Nottingham). Cells were used between passages sixty six and seventy.

2.1.3.3 Rat brain capillary endothelial cell line (rBCEC4)

The rat brain capillary endothelial cell line rBCEC4 was obtained from I. E. Blasig (Forschungsinstitut für Molekulare Pharmakologie, Berlin; Germany). Cell line rBCEC4 was used between passages twenty and twenty five.

2.1.3.4 Macrophage cell line (U937)

Macrophages cell line (U937) was received from the American Type Culture Collection (ATCC) (CRL-1593.2). Cells were used between passages seven and eleven.

2.1.4 Medium of tissue culture experiments

2.1.4.1 Growth medium for human colonic carcinoma epithelial (Caco-2) cell line

The growth medium was prepared by using 500ml minimum essential medium (M4655, Sigma) mixed with 10% fetal calf serum (F7524, Sigma), 1% non-essential amino acid solution (100x, M7145, Sigma) and 1% penicillin-streptomycin mixture (10.000 units penicillin and 10mg streptomycin/ml) (P4333, Sigma).

2.1.4.2 Infection medium for Caco-2 cell line

To prepare the Infection media, 500ml minimum essential medium (M4655, Sigma), was mixed with 10% fetal calf serum and 1% non-essential amino acid solution (100x, M7145, Sigma).

2.1.4.3 Growth medium for human brain microvascularendothelial cells (HBMEC) and rat brain capillary endothelial cell line (rBCEC4)

Dulbecco's modified Eagle's medium-high glucose DMEM (D6429, Sigma) 500ml was prepared and supplied with 10% fetal calf serum (F7524, Sigma), 1% non-essential amino acid solution (100x, M7145, Sigma) and 1% penicillin-streptomycin mixture (10.000 units penicillin and 10mg streptomycin/ml) (P4333, Sigma).

2.1.4.4 Infection medium for HBMEC and rBCEC4

500ml Dulbecco's modified Eagle's medium-high glucose DMEM (D6429, Sigma) was mixed with 10% fetal calf serum and 1% non-essential amino acid solution (100x, M7145, Sigma).

2.1.4.5 Growth medium for macrophage cell line (U937)

The growth medium was prepared by using 500ml RPMI-1640 medium (R8758, Sigma) supplied with 10% fetal calf serum (F7524, Sigma), 1% non-essential amino acid solution (100x, M7145, Sigma) and 1% penicillin-streptomycin mixture (10.000 units penicillin and 10mg streptomycin/ml) (P4333, Sigma).

2.1.4.6 Infection medium for macrophage cell line (U937)

500ml RPMI-1640 Medium (R8758, Sigma) was mixed with 10% fetal calf serum and 1% non-essential amino acid solution (100x, M7145, Sigma).

2.1.5 Buffers and detergents

2.1.5.1 Dulbecco's Phosphate Buffered Saline (PBS)

Phosphate buffered saline containing sodium and magnesium chloride solution (D8662, Sigma) was used to wash cells during preparation. The phosphate buffered saline was used for all cell lines used in this study.

2.1.5.2 Triton- X 0.5%

The solution of 0.5 % Triton-X was prepared by adding 500µl of Triton-X (Fisher BP151-100 Canada) to 99.5ml of sterile distilled water.

2.1.5.3 TE Buffer

The TE Buffer solution was prepared by using 5ml of 1M Tris base (BP152-500, Thermo Fisher Scientific), with 1ml of 0.5M EDTA , and 494ml distilled water. This buffer was used to prepare the plug agarose and to wash PFGE plugs.

2.1.5.4 Tris base- Boric acid – EDTA (TBE) Buffer 10X preparation (1 Litre distilled water)

The mixture was prepared using 108g Tris base (BP152-500, Thermo Fisher Scientific), 55g boric acid (B/3750/53), 40ml 0.5M EDTA pH 8.0 and autoclaved for 20 minutes.

2.1.5.5 TAE 1X

To make up TAE 1X 20ml of TAE 50X (2.0M Tris Acetate + 100 mM Na_2EDTA) were mixed with 980ml of distilled water.

2.1.5.6 Cell Suspension Buffer

To prepare a cell suspension buffer total volume = 3ml* n, Buffer needed as follows:

Tris pH 8.0 = 0.3ml* n

EDTA pH 8.0 = 0.6ml* n

VdH2O = 3ml* n – (V add Tris +V add EDTA)

n indicates the number of samples and V indicates Volume.

2.1.5.7 Cell lysis buffer

To prepare the cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl); 1% Sarkosyl NL (N-Dodecanoyl-N-methylglycine sodium salt) Sigma Aldrich, UK (L-5125), proteinase K 20mg/ml Sigma Aldrich, UK (P2308) and Calculate the total volume of cell lysis buffer/Proteinase K Buffer needed as follows:
total volume = 5ml *n
Tris, pH 8.0 = 0.25ml * n
EDTA, pH 8.0 = 0.5ml * n
Sarkosyl NL = 0.05g *n
proteinase k = 25µl * n and
VdH_2O = 5ml * n – (V Tris + V EDTA + V proteinase k)
(n indicates the number of samples and V indicates Volume).

2.1.5.8 Iron III solution

To prepare iron III solution, 9ml of HCl (100ml of distilled water added to 830µl HCl) was mixed with 1ml of $FeCl_3\,6H_2O$ solution (1L distilled water mixed with 2.73g of $FeCl_3\,6H_2O$).

2.1.5.9 Chrome azurol sulphate (CAS) solution

ChromeazurolS (199532, Sigma) was prepared by mixing 100ml of sterile distilled water with 0.0121g of CAS.

2.1.5.10 Hexadecyltrimethylammonium bromide (HDTMA)

Distilled water (80ml) was mixed with 0.1458g of HDTMA (H6268, Sigma).

2.1.5.11 Sodium hydroxide solution

Ten grams of 50% w/v NaOH was added to distilled water (20ml).

2.1.6 Molecular studies

2.1.6.1 Genomic DNA extraction

Each bacterial strain was grown aerobically in TSB broth with shaking, at 37°C incubator. The DNA was carried out with the GenElute™ Bacterial Genomic DNA Kit (Sigma) for each strain. Nanodrop 2000 (Thermo Scientific, UK) was used to check the concentration and purity of the eluted DNA.

2.1.6.2 PCR product purification

MinElute PCR Purification Kits (Qiagen, UK) were used to purify the amplified products by following the manufacturer's protocol. Nanodrop 2000 (Thermo Scientific, UK) was used to check the concentration and purity of the samples. The final elution step was conceded out in 20µl of molecular biology grade water (Fisher Scientific, UK). The sequencing machines were by Accugenix, Delaware (USA), Eurofins MWG Operon (London, UK) and Source Bioscience (Nottingham, UK). The data was analysed by Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.1.6.3 Agarose gel electrophoresis

The amplified PCR products were checked by visualisation on a 1% agarose gel (Life Science ® Company, UK, BIO-41025). The gel, 1% (w/v) agarose was mixed in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK) and microwaved until melted. From SYBR® Safe DNA gel stain (Life Technologies–Invitrogen, UK) 0.1µ/ml (v/v) was added to the molten gel and then poured in a tray and allowed to set. The gel was loaded with 5µl of sample and 100 bp DNA ladder (Promega, Southampton, UK) was used as a marker. The gel was run for 45 minutes at 90V in 1X TAE buffer. Under ultraviolet (UV) light, the gel was viewed to observe the DNA bands, using the InGenius® gel documentation system (Syngene; UK).

2.1.7 Safety considerations

Health and safety codes of practice were followed for all experiments. All materials used in the protocols were risk assessed using the suitable control of substances hazardous to health (COSHH) forms. Good laboratory practices were carried out for microbes, media and chemicals, as well as for operating laboratory devices. The Hepatitis B vaccination was received before commencement of tissue culture experiments.

2.2 Characterisation experiments

2.2.1 Phenotypic characterisation

The identification of the strains was initially determined using the ID32E (bioMérieux) phenotyping kit, according to manufacturers' recommendations, and the apiweb database (www.apiweb.biomerieux.com).

2.2.2 Genotypic characterisation

Pulsed-field gel electrophoresis (PFGE) was used as a technique for differentiating strains at the DNA level. The isolates were streaked on TSA and incubated at 37ºC for 14-18 hours. Each strain was suspended in 3ml cell suspension buffer (0.3ml Tris, 0.6ml EDTA and 2.1ml H_2O); section 2.1.5.6. The cell concentrations were adjusted to an optical density (OD) of 1.35 ± 0.05 at 610nm. The TE buffer was prepared and added

to 1% agarose and 1% SDS and kept at 55°C until required. Bacterial cell suspension (300µl) was mixed with 300µl of TEB agarose and 15µl of Proteinase K (20mg/ml, Sigma, P2308) and dispensed in 100µl volumes into each plug.

The plugs were placed in 50ml polypropylene tubes containing 5ml of cell lysis buffer/ Proteinase K (0.25 Tris, 0.50µl EDTA, 1% Sarcosyl and 25µl of Proteinase K); section 2.1.5.7 and left in a water bath shaker at 54ºC for 1.5-2 hours with vigorous shaking at 150-175 rpm. The lysis buffer was removed and 15ml of sterile ultra-pure water was added to wash the agarose plugs, and incubated in the water bath with shaking at 50°C for 15 minutes. This was repeated three times. The TE buffer (5ml) was added to the plugs and incubated in the shaking water bath at 50°C for 15 minutes. The TE buffer was poured off and the previous step repeated three times. The plugs were stored at 4°C in 5-10ml sterile TE buffer until required.

For restriction digestion of the DNA, 2mm of the plug was cut off and placed it in an Eppendorff tube containing 135µl of sterile water and 15µl of buffer for 30 minutes at room temperature. The mixture was removed and 150µl of restriction enzyme mixture (131.25µl sterilised water, 15µl TB buffer and 3.75µl enzyme Xba1) was added and incubated at 37°C for 1.5-2 hours. Each plug was put in order in the comb and 100ml of 0.5 TBE buffer (95ml H_2O, 5ml TBE and 1g agarose) were poured into the gel support tray.

Two litres prepared from the TBE buffer 0.5x (1900ml H_2O, 100ml TBE) was added to the running tank. The gel was run at 14°C with pulse times of 2.2 to 63.8 second for 20 hours at 6 V. After the electrophoresis run was over, the gel was stained by 20µl of ethidium bromide (Sigma, E1510) stock solution (10mg/ml) with 400ml of distilled water for 20-30 minutes in a covered container. The InGenius® gel documentation system (Syngene; UK) was used to photograph the gels. The electronic images of the PFGE patterns were saved in TIFF file format and analysed with Bionumerics software version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The *Salmonella* serotype Braenderup reference standard (H9812) strain 732 was used as the marker.

The tolerance value is expressed as a percentage of the total normalized gel length. Two bands on different PFGE patterns are considered to be the same if the difference in the position of the bands is less than the tolerance percentage. If the difference in the positions of the bands is greater than the tolerance percentage, then the bands are considered to be two different bands. A low tolerance (0.5%) allows for a minimal amount of shifting of the bands when comparing bands of different PFGE patterns. In this study different tolerance values were used when analyzing the PFGE patterns however the optimum band similarities were obtained at the tolerance values of 1.50%, 1.75% and 1.60% for EFT, MBM and Jordan strains respectively.

2.3 Identification of strains by using 16S rDNA sequence analysis

2.3.1 Preparation of FTA® Elute cards

The FTA Elute card was labelled with the sample code, and 5ml of sterile water was dispensed into a labelled tube. The suspension of bacteria was equal to 0.5 McFarland standard and 40µl of the suspension distributed in concentric circular motion within each circle. The dry cards were placed in an envelope and shipped to Accugenix, Inc.Delaware, USA.

2.3.2 AccuPRO-ID Bacterial Identification

Cultures were 24-48 hours old on TSA after incubation at 37°C as according to the recommended methodology from AccuPRO-ID bacterial proteotypic identification service. Each sterile Eppendorf tube (1.5ml) was labelled for each sample and 300µl of sterile, nuclease free water was placed in it. Each Eppendorf tube was inoculated with 1µl loop of bacteria from culture (24-48 hours old). Pure ethanol (900µl) was added to each suspension and the cap secured in preparation for shipment.

2.3.3 PCR 16S rDNA sequence analysis

Genomic DNA extraction was prepared as described above in section 2.1.6.1. The partial sequencing of the 16S rDNA loci (528 bp) was performed using primers described by Iversen *et al.* (2006). The PCR reaction mixture contained 5µl buffer, 2µl $MgCl_2$, 1µl 2'-deoxynucleoside 5'-triphosphate (dNTP), forward (TGGAGAGTTTGATCCTGGC-

TCAG) primer and reverse (TACCGCGGCTGCTGGCAC) primer (2.5µl each), 0.25µl TAQ polymerase, 1µl DNA and 10.75µl distilled water. PCR conditions were as follows: initial denaturation at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 30 second, primer annealing at 62.6°C for 30 second, extension at 72°C for 45 second; followed by a final extension step of 72°C for 10 minutes.

2.4 Physiological experiments

2.4.1 Heat tolerance

Overnight cultures were inoculated into whey-based ready to use formula (Cow and Gate First Infant Milk) and casein (SMA Extra Hungry Ready to use) and incubated at 37°C overnight. Infant milk was warmed to 55°C for 30 minutes in a water bath. Bacterial suspensions (1ml) were inoculated into 9ml warmed milk. At zero time 100µl of suspension were transferred to one well of the 96 well plates. This was repeated every 5 minutes for 30 minutes. Serial dilutions of the bacterial suspension (15µl) 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 55ºC. The plates were incubated at 37ºC overnight. The viable count was plotted against time, and D-values calculated on the time required for a 1 $\log_{10}$ reduction in the viable count.

2.4.2 Biofilm formation

Bacteria production was determined according to the method described by Stepanović *et al*. (2004), with a slight modification. In brief, bacteria were grown at 37°C overnight without shaking for 24 hours in TSB and then diluted to 10^4 cfu/ml in sterile saline and used to inoculate reconstituted formula (casein, whey and soya). Serial dilution of each type of inoculated formula milk was carried out into saline in the wells of a 96-well flat bottomed polystyrene microplate. The microplates were incubated aerobically for 24 hours and 48 hours at 20ºC and for 24 hours at 37ºC. The contents of each well were removed using a vacuum and the remaining biofilm was washed two times with 300µl of sterile saline and shaken for 15 minutes at 200 rpm. Crystal violet 0.01% (250µl) was added to the wells for 5 minutes, and then the microplates were washed two times with 250µl sterile distilled water per well. The residual attached bacteria were extracted with

200µl of ethanol (70% v/v) per well, and after 15 minutes 150µl was transferred to new plates.

The absorbance of each well was measured at 540nm using a GEN5 program, the triplicate results were averaged and divided by the negative control. The relative increase in biofilm formation level for each strain was obtained from dividing the OD of the strain retained by the isolates. The OD of tested strains was determined according to the method described by Stepanovic *et al*. (2004) where the threshold for positive bacterial biofilm OD was defined as three standard fold increase above the mean OD. of the negative control. Strains were categorised as follows: No biofilm formation: OD strain = OD control, low biofilm formation: OD strain ≤ 2x OD control, moderate biofilm formation: OD strain ≤ 4 x OD control and high biofilm formation: OD strain >4 x OD control.

2.4.3 Capsule formation

The preparation of milk agar media was described in section 2.1.2.3. Capsule production on milk agar by tested strains was determined by colony appearance on milk agar at 20ºC (after 24 hours and 48 hours) and 37ºC (after 24 hours).

2.4.4 Acid tolerance of organisms to pH 3.5 (HCl acidified formula)

Bacteria resistance to pH 3.5 was studied according to the method as already used by Edelson-Mammel *et al.* (2006), with some modification. A single colony of the test organism was inoculated into 5ml of TSB and incubated for 18 hours at 37°C. Two types of formula were used in this study (whey and casein-based formulas). The milk was adjusted to pH 3.5 with 1mM of sterile HCl (sterilised by filtration using 0.2µm filters). Two sterile tubes containing 9ml of pH adjusted formula were placed in a water bath at 37°C to avoid the heat shocking of the inoculated cells.

The pH adjusted infant formula was inoculated with 1ml of the bacterial cell suspension into the water bath to maintain the temperature. From each tube 100µl of adjusted infant formula was taken at each time point (0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes) and then were serially diluted in normal saline and plated on TSA agar plates by using

the Miles and Misra technique to determine the number of viable cells. The plates were incubated for 18 hours at 37°C and the number of colonies was enumerated as colony forming units (cfu). The number of colonies was transformed to log (cfu/ml) values for presentation on the log curve graph.

2.5 Virulence factors

2.5.1 Bacterial attachment and invasion of host cells

2.5.1.1 Preparation of bacterial inoculum

The strains were grown overnight on TSB at 37°C, The overnight culture were then pelleted by centrifugation for 3 minutes at 1200 rpm. The supernatant was then discarded and the pellet was re-suspended in 1ml of infection medium. Each strain was added at ~ 10^6 cfu per well to obtain the multiplicity of infection of 1:100 and incubated with 5% CO_2 at 37°C for 3 hours.

2.5.1.2 Mammalian cell culture

All cell lines were taken from the liquid nitrogen that was used for each of the tissue culture studies. The frozen ampoule was melted and quickly seeded into 10ml of the suitable growth medium, and 100µg/ml gentamicin was added to the initial passage. Bacterial attachment and invasion of host cells were determined by the method of Townsend *et al*. (2008b). The seeded cells were incubated at 37°C with 5% CO_2. The cells were centrifuged for 5 minutes at 1500 rpm by using CENTAUR2 centrifuge (MES, UK). The pellet was re-suspended in 25ml of growth medium and seeded into 75ml tissue culture flasks. The seeded cells were incubated at 37°C in 5% (v/v) CO_2, for 24 hours for rBCEC4 and HBMEC for 48 hours for Caco-2 and U937 to obtain the confluent layer (70-80%). Mammalian cells were then seeded in 24-well microplate for 48 hours and the concentration in each well was 2 x 10^4 cells/ml. Five millilitres of trypsin-EDTA solution was used to detach the confluent monolayer.

2.5.1.3 Attachment assay

The attachment and invasion assay was used to determine the ability of the bacterial strains to adhere Caco-2, rBCEC4, and HBMEC cell lines. To obtain less variation between the two assays, the determination of attachment was carried out at the same time using the same cell line passage of mammalian cell line and same inoculum of bacterial suspension. Cultivation of the Caco-2, rBCEC4, and HBMEC cell lines was described in section 2.1.3. The mammalian cells were washed three times using Dulbecco's phosphate buffered saline (PBS) (D8662, Sigma) and were infected with each isolate at ~ 10^6 cfu per well (MOI of 1:100) and incubated with 5% CO_2 at 37°C for 3 hours. After incubation, the cells were washed three times with PBS, and then 100µl 0.5% Triton-X100 was added to the wells and diluted with PBS to determine the viable count using the Miles and Misra method.

This assay enumerated the total number of bacteria associated with the mammalian cells after a 3 hours incubation period. These cells may be either attached to the surface or located intracellularly. Therefore, the number of attached bacteria was determined by subtracting the number of intracellular bacteria following invasion using the gentamicin protection assay. The average number of bacterial cfu/well was used to present data, given that each well contained 2 × 10^4 mammalian cells. The attachment efficiency of the tested isolates was determined by the number of bacteria attached per well/inoculum size ×100. The experiment was repeated twice in triplicate. Error bars in Figures indicate the standard deviation value.

The attachment and invasion assays were performed with the breast milk and nasogastric EFT isolates as listed in Table 2.5. *E. coli* 1230 and *Sal.* Enteritidis strain 358 were used as negative control and positive control respectively for Caco-2 cell line, whereas *Cit. koseri* strain 48 was used as positive control for rBCEC4 and HMBEC cell lines.

2.5.1.4 Gentamicin protection invasion assay

For the invasion assay, the mammalian cell lines Caco-2, rBCEC4, and HBMEC were used as described in section 2.1.4. These cells were washed three times with PBS and were infected with the organism at 2 x 10^6 cfu per well (MOI of 1:100) and incubated

with 5% CO_2 at 37°C for 3 hours. After incubation, the cells were washed three times with PBS and then 500µl of warm tissue culture media with 125µg/ml gentamicin (G1397, Sigma) were added to each well and incubated for 1 hour. Triton-X100 solution (100µl) was added to each well after removing the liquid and washing three times with PBS. Serial dilutions were carried out of each sample for enumeration following the Miles and Misra method on TSA plates. The invasion efficiency of tested strains was calculated by dividing the number of the internalised bacteria per well (cfu) by the initial bacterial cell number (cfu) and multiplying by 100. The experiment was repeated twice in triplicate. Error bars in figures indicate the standard deviation.

2.5.1.5 Gentamicin protection assay for U937 macrophage uptake and persistence

The ability of bacterial strains to internalise and persist in human macrophages was studied. The macrophage cells were cultivated in RPMI 1640 medium with 2mM L-glutamine, and modified to contain 10mM HEPES, 1mM sodium pyruvate, 4.5g/L glucose, 1.5g/L sodium bicarbonate, and supplemented with 10% foetal bovine serum. At 37°C and under 5% CO_2, the cells were treated with 0.1µg/ml of phorbol 12-myristate 13-acetate (PMA; Sigma) and placed in tissue culture plates to adhere and become activated. The period before infection was at least 24 hours.

RPMI medium was added prior to infection for washing the cells to remove residual PMA. The strains were grown overnight in TSB, and then 120µl was transferred to 5ml of fresh RPMI 1640 medium. The suspension was incubated for 2 hours at 37ºC in a shaking incubator at 160 rpm. The OD was measured by using a spectrophotometer (JENWAH, UK) and adjusted at 600nm to between 0.3-0.5nm. Bijoux bottles containing 3ml of tissue culture media were inoculated with bacteria with a final OD of 0.005nm. The bacteria were inoculated at ~ 10^5 cfu per well and U937 human macrophages were seeded at 2×10^4 cells per well to obtain the multiplicity of infection of 1:10 for 45 minutes at 37°C in 5% CO_2 (v/v) incubator. The cells were then washed three times with PBS.

The persistence of bacterial cells in macrophages up to 48 hours was investigated by the maintenance of two sets that were infected for 45 minutes. After the incubation period,

the macrophages were resuspended in U937 medium supplemented with 100μg/ml and 10μg/ml of gentamicin for 24 hours and 48 hours respectively and incubated at 37°C in 5% CO_2. Macrophages were washed three times with PBS and then lysed with 0.5% Triton-X100, pipetted to homogenise, decimally-diluted and then plated on TSA to determine the number of intracellular bacteria by using the Miles and Misra technique. The uptake and persistence efficiency of bacterial strains was calculated by using the number of the internalised bacteria per well (cfu) divided by the total of bacterial cells (cfu) in the inoculum and multiplying by 100.

The U937 macrophage uptake and persistence assays were performed with the MBM and nasogastric EFT isolates as listed in Table 2.5. *E. coli* strain 1230 and *Cit. koseri* strain 48 were used as negative control and positive control respectively. The standard deviation is indicated by the mean of uptake and persistence efficiency of each isolate for two independent experiments in triplicates.

2.5.1.6 The adhesion patterns of bacteria with Caco-2 human epithelial cells

Six well microplates were seeded with 2 x 10^4 Caco-2 cells and then incubated at 37°C with 5% CO_2 for 48 hours and checked for adherence and confluent growth. Overnight bacterial cultures were centrifuged and resuspended in 8ml of appropriate complete medium DMEM. The tissue culture cells were infected with ~ 10^8 bacteria per well (4ml) giving a MOI 1:10000. The infected Caco-2 cells were incubated at 37°C with 5% CO_2 for 3 hours. The medium was aspirated after the incubation period and the cells washed three times with sterile PBS. The coverslip for control slide was prepared by removing the medium immediately from the chamber slide, then the coverslip was removed and the cells washed three times with 5ml sterile PBS.

Absolute methanol was used for 5 minutes to fix the slides, and these were air-dried. Giemsa staining was performed following a modified method described by Mange *et al*. (2006). The Giemsa stain was diluted 1: 20 (5%) with distilled water, then the cells were stained for 15 minutes, then washed with distilled water and allowed to air dry. The slides were viewed using light microscopy under oil immersion.

2.5.2 Haemolysin production

Tryptic soy agar (500ml) was prepared according to the manufacturer's instructions and sterilised at 121°C for 15 minutes. After cooling to 45-50°C, 25ml of sterile sheep blood defibrinated (Oxoid, SR0051) or horse blood (Oxoid, SR0050) were added to create a 5% suspension of sheep or horse blood and mixed gently and dispensed into sterile Petri dishes. A single colony was harvested from a TSA plate and streaked onto blood agar plate, and incubated for 18 hours at 37°C. Following the haemolytic reaction on a blood agar plate, strains were categorised as follows: α-partial haemolysis as greenish colour around the colonies, ß-indicated by clearing around colonies on blood agar and γ - indicated by a lack of clearing or another change around colonies on blood agar. *S. pyogenes* NCTC 9994 was used as positive control.

2.5.3 Serum sensitivity determination

Serum resistance was determined according to the method described by Hughes *et al*. (1982), with a slight modification. Serum was obtained from human male AB plasma (Sigma, H4522). The strains were grown overnight in LB Broth and were centrifuged (1500g for 5 minutes), the pellet was diluted to $l0^6$ cfu/ml in 5ml of phosphate buffered saline. An inoculum (0.5ml) of suspension cells was added to 1.5ml of undiluted serum, equal volumes (0.2ml) of this suspended and human serum were mixed before incubation at 37°C.

Viable counts were obtained at the beginning and after 1, 2, 3 and 4 hours of incubation at 37°C. Serially diluted cells were plated on TSA agar plates using the Miles and Misra technique and incubated aerobically at 37°C for 18 hours before the number of colonies was counted. The experiment was in duplicate. *E. coli* strain 1230 and *Salm.* Enteritidis strains 358 were used as negative control and positive control respectively.

2.5.4 Siderophore production

For siderophore production the method of Gundogan *et al*. (2011) was used with slight modification. Chrome azurolsulphate (CAS) agar was prepared by using two solutions. The first solution (dark blue liquid) was prepared by using 50ml of CAS solution (see

section 2.1.5.9), 10ml of iron III solution (section 2.1.5.8) and 40ml of HDTMA before autoclaving at 121°C for 15 minutes (100ml in total of dark solution). The second solution was prepared by mixing 900ml of distilled water, 15g agar, 30.24g pipes and 12g NaOH and then autoclaving at 121°C for 15 minutes. After autoclaving, the first solution was mixed with the second solution and then the media was poured into the plates.

Immediately before use, 5mm diameter holes were punched into the agar. To prepare the bacterial suspension, five colonies were taken from TSA and inoculated into LB broth containing 200µM of 2,2´-dipyridyl (31.236mg in 1L of LB broth) and incubated with shaking (170 rpm) at 37°C overnight. After incubation, the sample was centrifuged at 5000 rpm for 10 minutes and 70µl of the supernatant was added into the holes. The agar was incubated at 37ºC for 4-8 hours. The observance of an orange zone around the hole indicated that the strain was positive for siderophore production. *Y. enterocolitica* strain 8081 was used as a positive control.

2.5.5 Determination of high pathogenicity island

Each bacterial strain was grown aerobically in TSB broth with shaking, at 37°C incubator. The DNA was carried out with the GenElute™ Bacterial Genomic DNA Kit (Sigma) for each strain. Amplifications were done in a 25µl reaction 2 m*M* $MgCl_2$, 1µl Deoxyribonucleotide Triphosphate (dNTPs) mix, 1µl of each primer, and 0.25 U of Taq Flexi DNA polymerase (Promega, UK). The method was as previously described by Schubert *et al*. (2000). The initial denaturation step (94°C, 5 minutes) was followed by 30 cycles of denaturation (94ºC, 1 minute), annealing (annealing temperature, 1 minute), and extension (72ºC, 1 minute) with 1 final extension step (72ºC, 8 minutes). The sequences of the forward primers and reverse primers, the annealing temperature, and the size of the amplified fragment are presented as follows:

- *irp1* primer forward TGAATCGCGGGTGTCTTATGC and reverse TCCCTCAATAAAGCCCGCT (product size 238 bp; temperature, 57ºC).
- *irp2* primer forward AAGGATTCGCTGTTACCGGAC and reverse TCGTCGGGCAGCGTTTCTTCT, (product size 287 bp; temperature, 57ºC) and;

- *fyuA* primer forward GCGACGGGAAGCGATGATTTA and reverse TAAATGCCAGGTCAGGTCACT (product size 547 bp; temperature, 56ºC).

Samples were electrophoresed in 1.5% agarose gels which were stained with SYBR® Safe DNA Gel Stain, and a 100 bp DNA ladder (Promega, UK) to determine product size [Appendix 2]. The running tank was filled with 1X TAE buffers and gels were run for 45 minutes at 90 volts. Under the UV light, using the InGenius® gel documentation system (Syngene; UK), DNA samples were visualized. *Y. enterocolitica* strain 8081 was used as positive control.

2.5.6 Antimicrobial susceptibility testing and ESBLs detection

The disk diffusion method was used to determine bacterial susceptibility in accordance with standardised HPA (2006) and BSAC methods (2008) for the following antibiotics: amikacin (30µg), ampicillin (10µg), cefotaxime (30µg), ceftazidime (30µg), cephalothin (30µg), ciprofloxacin (1µg), gentamicin (10µg), imipenem (10µg), cefuroxime (30µg), chloramphenicol (30µg), streptomycin (10µg), ceftriaxone (30µg), cefoperazone (30µg), doxycycline (30µg), cefotetan (30µg), cefpodoxime (10µg), co-amoxiclav (20+10µg; zone diameter based on a 2:1 ratio of amoxicillin:clavulanate) and trimethoprim (2.5µg).

ESBLs production was determined by the combination of cefpodoxime (CPD) kit (30-µg) (Oxoid, DD0029) and cefpodoxime-plus-clavulanate (CD CV) (30- plus 10-µg) disks, ceftazidime (CAZ) (30-µg) and ceftazidime-plus-clavulanate (CAZ CV) (30- plus 10-µg) disks and cefotaxime (CTX) (30-µg) and cefotaxime-plus-clavulanate (CTX CV) (30- plus 10-µg) disks. From overnight cultures, 4-5 colonies were transferred into a tube containing 5ml of a sterile saline and mixed thoroughly via vortex to give just visible turbidity. The suspension was adjusted to the density of the 0.5 McFarland standards. ISO-sensitest agar (Oxoid, CM0471) was streaked by adjusted suspension of organisms. The disk tests were performed with confluent growth on ISO-sensitest agar (Oxoid, CM0471). After incubation for 18 hours at 37°C, a zone of inhibition can be observed. *E. coli* NCTC 10418 was used as a control organism as it is sensitive to all antimicrobial drugs. An ESBL was detected by measuring a difference of ≥ 5mm between the zones of combination disc compared to that of the individual antibiotic cefpodoxime/ceftazidime and cefotaxime.

2.5.7 Data analysis

In this study, two comparisons were analysed using ANOVA one way (Tukey method) analysis test and a P value of less than 0.05 ($p < 0.05$) was considered to indicate statistical significance. The first comparison was between biofilm levels in each type of infant formula (whey, casein and soya) at two temperatures 20°C and 37°C by each strain. Additionally, ANOVA one way (Tukey methods) analysis test was used in order to compare between levels of biofilm for the species in different types of formula and different temperatures. Furthermore, one way ANOVA also was used to find the consistence of the independent experiments for the strains to adhere Caco-2, rBCEC4, and HBMEC cell lines, the significance was set at $p < 0.05$. All experiments were formed in triplicate except for experiments of acid tolerance of organisms at pH 3.5 which were performed in duplicate. Data are presented as the average of two independent experiments. Error bars in figures represent the standard deviation.

Chapter 3: CHARACTERISATION AND IDENTIFICATION OF BACTERIAL ISOLATES

3.1 INTRODUCTION

There are a variety of phenotypic and genotypic methods to determine if isolates are indistinguishable or closely related. These tests include PFGE and multilocus enzyme electrophoresis, as described by Tenover *et al.* (1995). The most common technique, which is still considered the gold standard, for bacterial strain characterisation is PFGE. The PFGE technique is used by many laboratories to determine strain relatedness to identify the source of a strain or outbreak, and to confirm an outbreak of a bacterial disease (Agasan *et al.*, 2002; Bender *et al*., 2001). This technique relies on its ability to separate and visualise DNA molecules based on their size. In addition, PFGE has shown an excellent ability to separate DNA molecules ranging in size from a few kilobases (kb) to greater than 10 megabase pairs (Mb) (Steward *et al*., 1988; Burmeister *et al.*, 1992).

PFGE technique is able to separate large of DNA molecules by the use of an alternating electrical field, such that greater size resolution can be obtained when compared to normal agarose gel electrophoresis. PFGE is often employed to track pathogens, such as *Salmonella*, *Shigella*, *E. coli* (including O157) (Peters, 2009). However, current technology has been certain that whole-genome sequencing to be a highly discriminating and efficient method for strain typing of outbreak samples. it has been recently applied to subtyping analysis, and it bridges the gap between PFGE and whole-genome sequencing. Whole-genome sequencing uses restriction site analysis arranges 200 to 500 bands in the order they appear on the chromosome while PFGE utilizes ∼20 bands sorted by size. The most important limitation of whole-genome sequencing at the present time is the cost time in spent preparing longer than the time required for PFGE analysis, sequencing, and analysing samples (Miller, 2013; Pendleton *et al*., 2013).

The Centres for Disease Control and Prevention (CDC) and PulseNet possess standardized protocols to compare PFGE patterns for most foodborne pathogens, such as *Salmonella*, *E. coli* O157:H7, *Shigella*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Yersinia pestis* (CDC, 2004; Cooper *et al*., 2006).

There are several commercially available restriction enzymes, such as *Sma I*, *Spe I*, *Xba I*, *Nhe I*, *Apa I* and *Avr II*, with the ability to cleave DNA at specific sites on the genome

(Basim and Basim, 2001). The enzymes are regularly utilized to improve characterisation and discrimination between bacteria (Tenover *et al*., 1995). The restriction enzymes act to cut the genomic DNA into small fragments (8 – 25) which are separated by using agarose gel electrophoresis. Then the electronic images of PFGE ('tiff' format) can be analysed using commercially available software such as BioNumerics (CDC, 2004).

To interpret the PFGE patterns in epidemiological investigations for the analysis of restriction patterns, the following criterion suggested, by Tenover *et al*. (1995), is often used:

- ***Indistinguishable:*** strains are considered indistinguishable if their restriction patterns have the same band numbers and those bands are identical in size.
- ***Closely related:*** strains have between one to three bands different from each other.
- ***Possibly related:*** isolates are considered possibly related if they are differ by four to six band differences.
- ***Unrelated strains:*** The isolates are considered unrelated if the strains differ by 7 or more bands.

Although phenotyping is a quick method for isolate identification, it is prone to the subjective reading of results, and dependence upon an external database. 16S rDNA sequence analysis is more reliable, although it requires a longer period of time before the results are obtained. In addition, because of its universal distribution among bacteria 16S rDNA sequences is a useful alternative when phenotypic characterisation methods fail. 16S rRNA gene sequences are used to study bacterial phylogeny and taxonomy for many reasons, specifically that all bacteria have a 16S rRNA gene, often existing as a multigene family or operons. Moreover, there is no change in the function of 16S rRNA as well as the large size of 16S rRNA gene (1500bp) which is really helpful in the bioinformatics analysis (Patel *et al.*, 2001).

This study used twenty bacterial strains isolated from mastic human breast milk by lactation consultants at different primary health-care centres in Spain (Delgado *et al.*, 2008). Initial studies were, therefore, necessary to confirm the identity of these strains

and to determine if any were clones, i.e., that same strain may have been isolated more than once. Hence, the strains were analysed using ID32E (phenotyping), pulsed-field gel electrophoresis and 16S rDNA sequence analysis. In addition, seventy five EFT isolates of *K. pneumoniae* (from Jordan) were also analysed, but only for PFGE profile due to time constraints.

Initial PFGE subtyping for one hundred and thirty-three strains isolated from neonatal EFT from two local Nottingham hospitals (QMC and NCH) was undertaken by Alkeskas (former NTU PhD student) as part of his research (see Figure 3.1-4). His study was taken further by myself and the PFGE fingerprinting was re-analysed and the strains were re-identified using 16S rRNA. While MBM strains and Jordan strains were analysed using PFGE technique by myself. DNA fingerprinting via PFGE for these strains was used to determine their genotypic similarities and the variation between isolates. Furthermore, this study investigated if the same strains were isolated from the same baby on more than one occasion, or from different neonates, and to identified if the same strains were isolated at different times from the NICUs. Previous research at NTU has also identified these strains by using ID20E, ID32E and 16S rDNA sequence analysis. The MBM strains were analysed by using ID32E, and 16S rDNA sequence analysis.

3.2 Materials and Methods

The preparation of culture media and conditions for this chapter were described previously in section 2.2 of chapter 2 'Materials and Methods'. Briefly, the dendogram was created by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The analysis of the PFGE data was interpreted using criteria proposed by Tenover *et al*. (1995).

3.3 Results

3.3.1 Characterisation of *Enterobacteriaceae* strains using the PFGE technique

The tolerance and optimisation for the bands were 1.50%, 1.75% and 1.60% for EFT strains, MBM strains and Jordan strains respectively. As according to a previous study by Hunter *et al*. (2005) *Salmonella* serotype Braenderup strain H9812 (NTU collection *Salmonella* strain 732) was chosen as the universal size standard; see [Appendix 1]. This

strain had coverage of a large range of DNA fragment sizes, even distribution of bands, and optimises stability of the PFGE pattern.

The universal size standard was used to establish reference positions (visible restriction fragments in the pattern), which are used to normalise the electronic images ('tiff' format). The protocol that was used in this experiment has been standardised by the CDC and PulseNet (2004).

3.3.1.1 Characterisation of enteral feeding tubes strains

One hundred thirty-three *Enterobacteriaceae* strains isolated from neonatal enteral feeding tubes from two local hospitals in Nottingham (NCH and QMC) were analysed. These were *Ent. hormaechei*, *Ent. cancerogenus*, *Klebsiella* spp., *Ent. cloacae* and *Ent. aerogenes*. As shown in Figure 3.1, forty four *Ent. hormaechei* strains formed seven distinguishable pulsotypes and two unique strains. Fifty one strains of *Ent. cancerogenus* formed 6 distinguishable pulsotypes and there were two unique strains, see figure 3.2. In Figure 3.3, thirty three *Klebsiella* spp. strains formed 6 distinguishable pulsotypes and one unique strain. Finally, the remaining species are presented in one Figure (Figure 3.4) which were three strains of *Ent. cloacae* and two strains of *Ent. aerogenes*. They were clustered into two distinguishable pulsotypes, and there is one which was unique.

➢ Characterisation of *Ent. hormaechei* strains

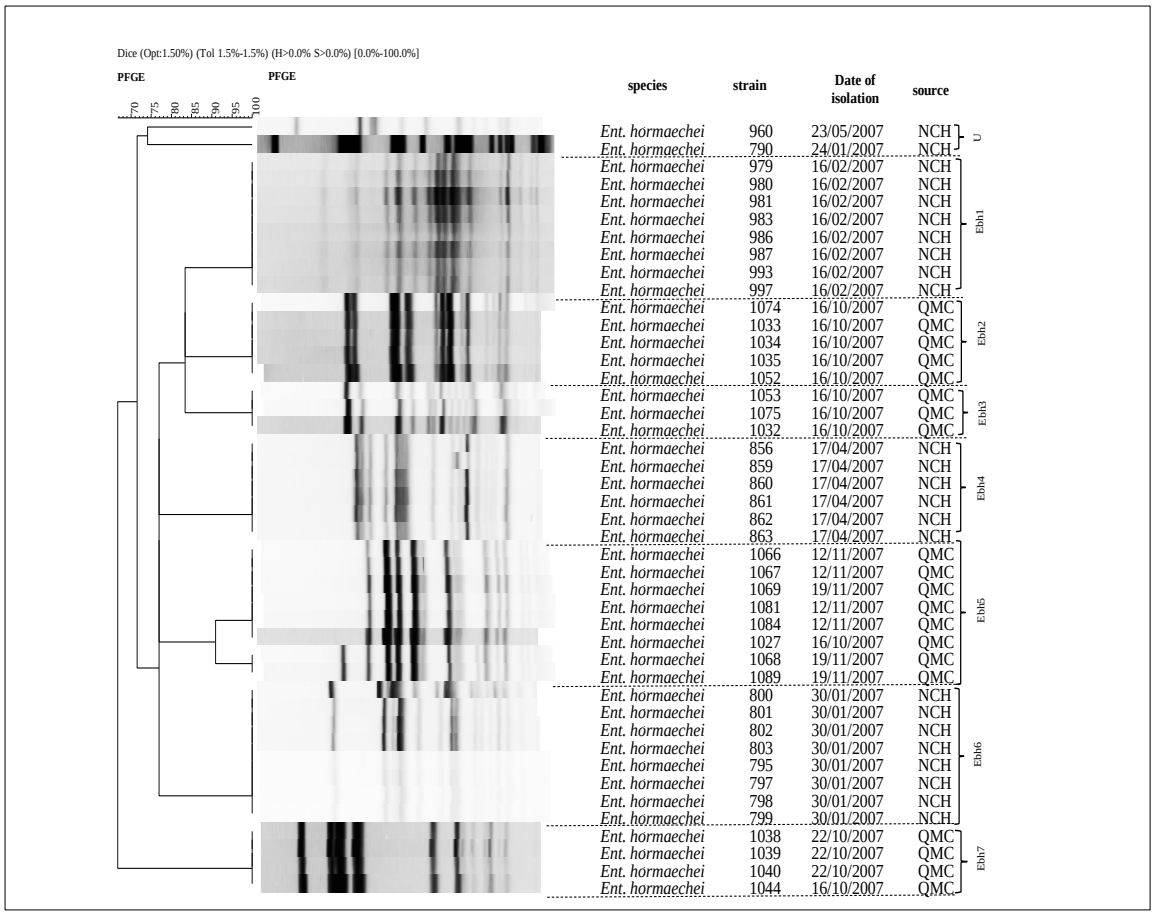


Figure 3.1: PFGE cluster analysis of *Ent. hormaechei* strains (as performed by Alkeskas).

The dendrogram was generated by BioNumerics softwar e, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%. Ebh1- Ebh7 and U: indicates PFGE cluster groups; U: Unique.

Table 3.1: Summary of PFGE analysis of *Ent. hormaechei* strains.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
Ent.hormaechei	NCH	790,960	U	2	Over four month	2
		979, 980, 981, 983, 986,987,993,997	Ebh1	8	same day	1
		856, 859, 860, 861, 862,863	Ebh4	6	same day	3
		795, 797, 798, 799, 800,801,802,803	Ebh6	8	same day	6
	QMC	1052, 1033, 1034, 1035, 1074	Ebh2	5	same day	2
		1032, 1053, 1075	Ebh3	3	same day	2
		1027, 1066, 1067, 1081, 1084, 1068,	Ebh5	8	Over one month	3
		1044, 1038, 1039, 1040	Ebh7	4	one week	2

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital.Ebh1- Ebh7 indicates PFGE cluster groups, U: Unique.

Table 3.1 and Figure 3.1 present the analysis of forty four EFT strains of *Ent. hormaechei* which previously have been isolated from two local hospitals; NCH (n = 24) and QMC (n = 20) [Appendix 3]. The strains were obtained from 19 patients. These strains were clustered into seven pulsotypes; Ehc1- Ehc7 and two unique strains. All strains in pulsotypes Ebh1-Ebh4 and Ebh6- Ebh7 showed a 100% similarity coefficient.

From the same patient, 8 isolates of *Ent. hormaechei* were recovered which belonged to pulsotype Ebh1. These strains were isolated in one day (16th February 2007).

Pulsotype Ebh2 was composed of five strains of *Ent. hormaechei*. They were isolated from 2 patients, from same hospital (QMC) on the same day (16th October 2007). Strains 1033, 1034 and 1035 were isolated from the same patients and, strains 1052 and 1074 were from another patient.

Three strains of *Ent. hormaechei* belonged to pulsotype Ebh3, which were isolated on the same day (16th October 2007). These strains were isolated from two different neonates in the same hospital. Two strains, 1053 and 1075, were isolated from the same patient while strain 1032 was isolated from a different patient.

Six strains of *Ent. hormaechei* were grouped into pulsotype Ebh4. These strains were isolated from three different neonates in one day (17th April 2007). They were in the

same hospital. Four strains, 860-863, were isolated from the same patient whereas strains 856 and 859 were isolated from two different patients.

A total of eight *Ent. hormaechei* strains belonging to pulsotype Ebh5 were isolated during a period of one month (16^{th} October and 12^{th} and 19^{th} November 2007) from 3 different patients in the same hospital (QMC). Pulsotype Ebh5 was composed of two sub-clusters. The similarity coefficient of each sub-cluster was 100% validating the clonality of the isolates in each sub-cluster. While the similarity coefficient of strains 1068 and 1089 with the other 6 strains (1066, 1067, 1069, 1081, 1084 and 1027) was 91% which again validates that these isolates are higly identical. The difference between these strains was only one band.

Eight *Ent. hormaechei* strains belonged to pulsotype Ebh6 which were isolated on the same day (30^{th} January 2007) from six different patients in the same hospital (NCH). Four strains out of eight strains were isolated from two different patients. Strains 795 and 797 were isolated from the same patient while strains 798 and 799 were isolated from another patient.

Four *Ent. hormaechei* strains were isolated during a one week period (16^{th} and 22^{nd} October 2007) and belonged to pulsotype Ebh7. These were isolated from 2 different patients in the same hospital (QMC). Three strains of pulsotype Ebh7 were isolated from the same patient on the same day (22^{nd} October 2007) while one strain was isolated from another patient on different day (16^{th} October 2007). The similarity coefficient of the cluster was 100%.

The remaining two *Ent. hormaechei* strains (790 and 960) were unique and were isolated from two different patients from the same hospital.

➢ *Characterisation of Ent. cancerogenus* strains

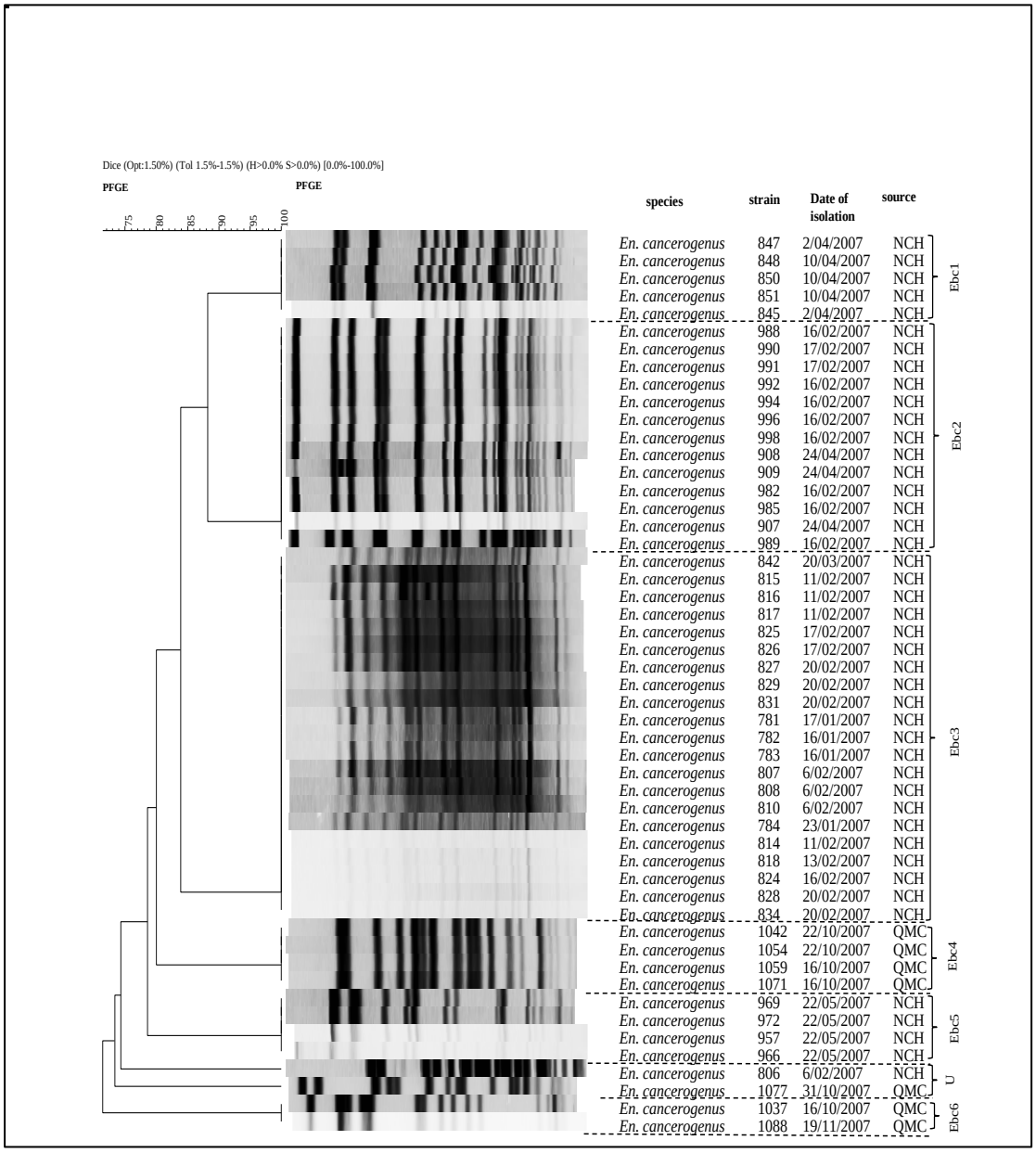


Figure 3.2: PFGE cluster analysis of *Ent. cancerogenus* strains (as performed by Alkeskas).

The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%. Ebc1- Ebc6 indicates PFGE cluster groups; U: Unique.

Table 3.2: Summary of PFGE analysis of *Ent. cancerogenus* strains.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
Ent. cancerogenus	NCH	845,847,848,850,851	Ebc1	5	over one week	2
		982,985,988,989,992,994,996,998,990, 991,907, 908, 909	Ebc2	13	About three months	3
		782, 783, 781, 784, 807,808,810,814,815,816,817,818,824,825,826,827, 828,829,831,834,842	Ebc3	21	over two months	13
		957, 966,969,972,	Ebc5	4	same day	3
		806	U	1	one day	1
	QMC	1077	U	1	one day	1
		1059, 1071, 1042, 1054	Ebc4	4	one week	4
		1037, 1088	Ebc6	2	over one month	2

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Ebc1- Ebc6 indicates PFGE cluster groups, U:Unique.

As displayed in Figure 3.2, the dendrogram of 51 isolates of *Ent. cancerogenus* from NCH (n = 44) and QMC (n=7), the strains have been obtained from 25 patients [Appendix 4]. These organisms were clustered into six distinguishable pulsotypes Ebc1-Ebc6 and two unique strains. The isolates of each pulsotype showed 100% similarity coefficient. All strains within each pulsotypes showed 100% similarity coefficient (Figure 3.2, Table 3.2).

Five strains of *Ent. cancerogenus* belonged to pulsotype Ebc1, which were isolated during 2 days (2nd and 4th April 2007) from the same patient.

A total of thirteen strains of *Ent. cancerogenus* were isolated over a period of two month (16th and 17th February and 24th April 2007) from 3 different patients in the same hospital (NCH).These strains belonged to pulsotype Eb2. There was a large number of strains isolated from the same patients; see Table3.2.

Twenty one strains of *Ent. cancerogenus* belonged to pulsotype Ebc3, which were isolated over a prolonged period of over two months (16th, 17 th and 23rd January, 6th,11th,13th,16th, 17th and 20th February and 20th March 2007) and the strains were isolated from 13 different neonates in the same hospital. Most strains in cluster Ebc3 were isolated from the same patient on the same day. Two strains 807 and 808 were isolated from the same patient while four strains 814, 815, 816 and 817 were isolated

from one patient. In addition, two strains 825 and 826 were isolated from the same patient. Also strains 827, 828, 829 and 831 were isolated from the same patients.

There were four strains of *Ent. cancerogenus* in pulsotype Ebc4, which were isolated in one week period (16th and 22nd October 2007) from 4 different patients in the same hospital (QMC).

Four strains of *Ent. cancerogenus* belonged to pulsotype Ebc5, which were isolated on the same day (22nd May 2007) and the strains were isolated from three different neonates in the same hospital. Two strains 969 and 972 were isolated from the same patient while strain 957 and 966 were isolated from different patients.

Pulsotypes Ebc6 was composed of two strains of *Ent. cancerogenus* isolated over a prolonged period of one month (16th October 2007 and 19th November 2007) isolated from 2 different patients in the same hospital (QMC).

Two strains (806 and 1077) were unique and were isolated from two different patients from different hospitals.

➢ *Characterisation of Klebsiella* spp. strains

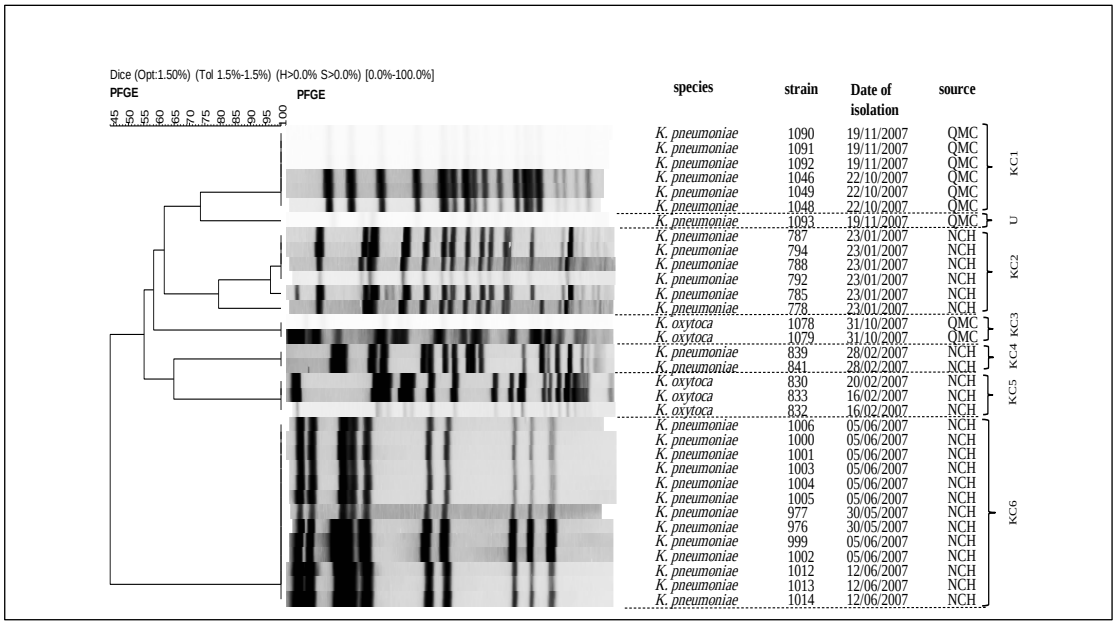


Figure 3.3: PFGE cluster analysis of *Klebsiella* spp. (as performed by Alkeskas).

The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%. Kc1- Kc6 indicates PFGE cluster groups; U: Unique.

Table 3.3: Summary of PFGE analysis of *Klebsiella* spp. strains.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
K. oxytoca	NCH	832, 833, 830	Kc5	3	about one week	2
K. pneumoniae		778, 785, 787, 788, 792,794	Kc2	6	same day	6
		839,841	Kc4	2	same day	1
		976, 977, 999, 1004, 1006,1000,1002,1005,1001,1003,1012,1013,1014	Kc6	13	two weeks	5
	QMC	1046, 1048, 1049, 1090, 1091,1092	Kc1	6	one month	2
		1093	U	1	one day	1
K. oxytoca		1078, 1079	Kc3	2	one day	2

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Kc1- Kc6 indicates PFGE cluster groups, U: Unique.

The strains of *Klebsiella* spp. were clustered into 6 pulsotypes isolated over a wide time period (Figure 3.3, Table 3.3). Thirty three strains of *Klebsiella* spp. had been isolated from NCH (n=24) and QMC (n=9) from 19 patients [Appendix 5]. These isolates were in 6 clusters, Kc1-Kc6 and one unique strain. The strains in pulsotypes Kc1 and Kc3-Kc6 displayed 100% similarity coefficient.

Six strains of *K. pneumoniae* belonged to pulsotype Kc1. These were isolated over three weeks (22nd October and 19th November 2007) and from 2 different neonates in the same hospital (QMC). Three strains; 1046, 1048 and 1049, were isolated from the same patient on the same day (22nd October 2007) while strains 1090, 1091 and 1092 were isolated from another patient on the same day (19th November 2007).

Pulsotype Kc2 was composed of six strains of *Klebsiella* spp. which were isolated on the same day (23rd January 2007) from 6 different patients in the same hospital (NCH).Six strains of pulsotypes Kc2 was composed of three sub-clusters: 4, 1 and 1. The similarity coefficient of one sub-cluster was 100% with strains 787, 794, 788 and 792 which were isolated in one day from 4 different patients. However, these strains had a similarity coefficient (97%) with strain 785 which was caused by a difference of one band. On other hand, these two sub-clusters had a similarity coefficient with strain 778 (80%).

From two different patients, 2 isolates of *K.oxytoca* were recovered which belonged to pulsotype Kc3. These strains were isolated on one day (31st October 2007).

Pulsotype Kc4 was composed of two strains of *K. pneumoniae* which were isolated in one day (28th February 2007) and were isolated from the same patient.

Three strains of *K.oxytoca* belonged to pulsotype Kc5, which were isolated 5 days apart (16th and 20th February 2007) and were from two different neonates in the same hospital. Two strains; 832 and 833, were isolated from the same patient on the same day while strain 830 was isolated from another patient.

Thirteen strains of *K. pneumoniae* belonged to pulsotype Kc6. There were isolated during two weeks (30th May and 5th and 12th June 2007) from five different neonates in the same hospital. Nine strains were isolated from 3 different patients; strains 999, 1004,

1006 from one patient, another three strains 1000, 1002 and 1005 were from the second and strains 1012, 1013 and 1014 from third patient. In addition, four strains were isolated from two different patients; 976, 977, 1001 and 1003.

One strain, 1093, was unique and was isolated from one patient.

➢ Characterisation of *Ent. cloacae* and *Ent. aerogenes* strains

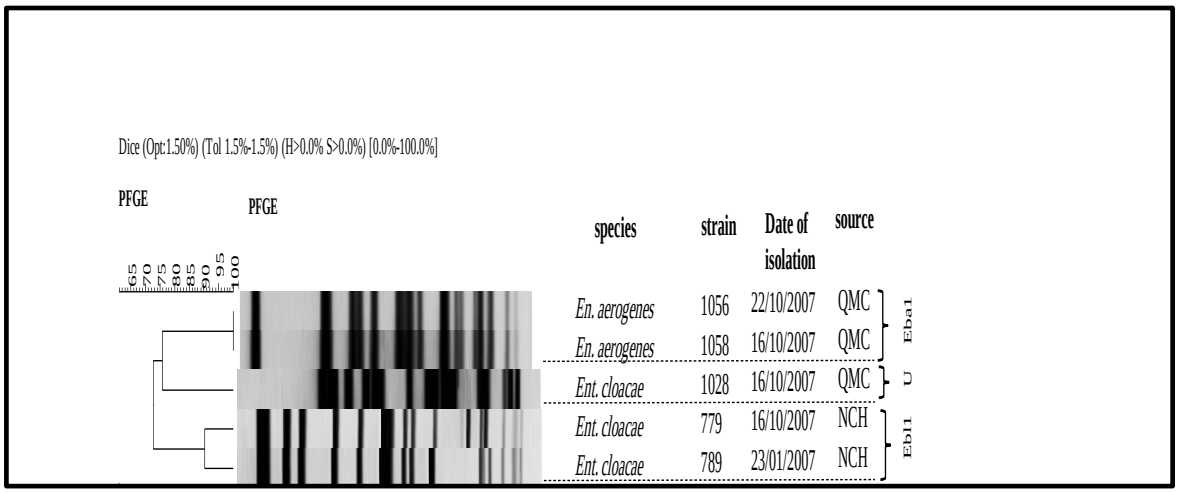


Figure 3.4: PFGE cluster analysis of *Ent. cloacae* and *Ent.aerogenes* (as performed by Alkeskas).

The dendrogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.50%. Eba1- Ebl1 indicates PFGE cluster groups; U: Unique

Table 3.4: Summary of PFGE analysis of *Ent. cloacae* and *Ent. aerogenes*.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
Ent. cloacae	NCH	779, 789	Ebl1	2	one week	2
Ent. aerogenes	QMC	1056, 1058	Eba1	2	one week	2
Ent. cloacae		1028	U	1	one day	1

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Ebl1 and Eba1 indicate PFGE cluster groups, U: Unique.

Table 3.4 summarises the results of Figure 3.4. The five different isolates of *Enterobacteriaceae* (*Ent. cloacae* and *Ent. aerogenes*) have been obtained from two local hospital NCH (n = 2) and the remaining 3 were from QMC [Appendix 6]. The strains were clustered into two pulsotypes; Eba1 and Ebl1 and two unique strains. Thier analysis is described briefly below.

From different patients 2 isolates of *Ent. cloacae* were recovered which belonged to pulsotype Ebl1. These strains were isolated in one week (16^{th} and 22^{nd}October 2007). They displayed 100% of similarity coefficient.

Two strains of *Ent. aerogenes* belonged to pulsotype Eba1, which were isolated during one week (16^{th} and 23^{rd} October 2007) and the strains were isolated from two different neonates in same the hospital. The similarity coefficient was 91%. The difference was one band.

Two strains (1028 and 1094) were unique and were isolated from two different patients from the same hospitals.

3.3.1.2 Characterisation of *Enterobacteriaceae* isolated from mastic breast milk

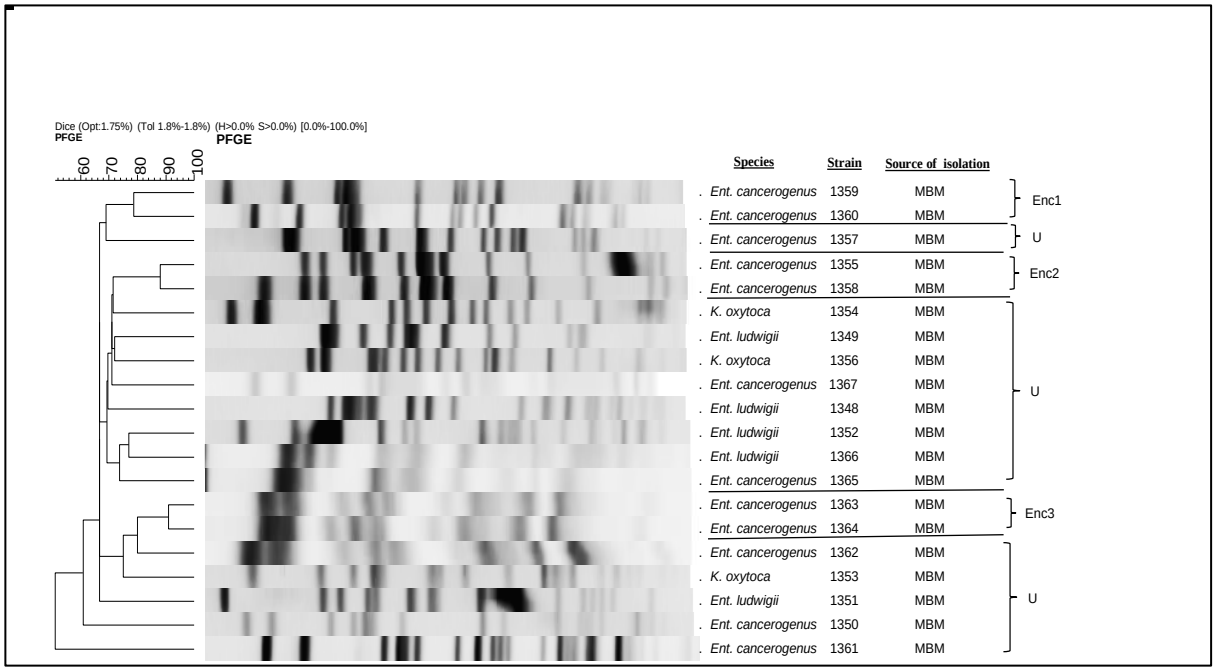


Figure 3.5: PFGE cluster analysis of MBM strains.

PFGE profiles of twenty bacterial strains isolated from mastic human breast milk. The dendogram was created by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.75%.U: Unique and closely related indicate PFGE cluster groups.

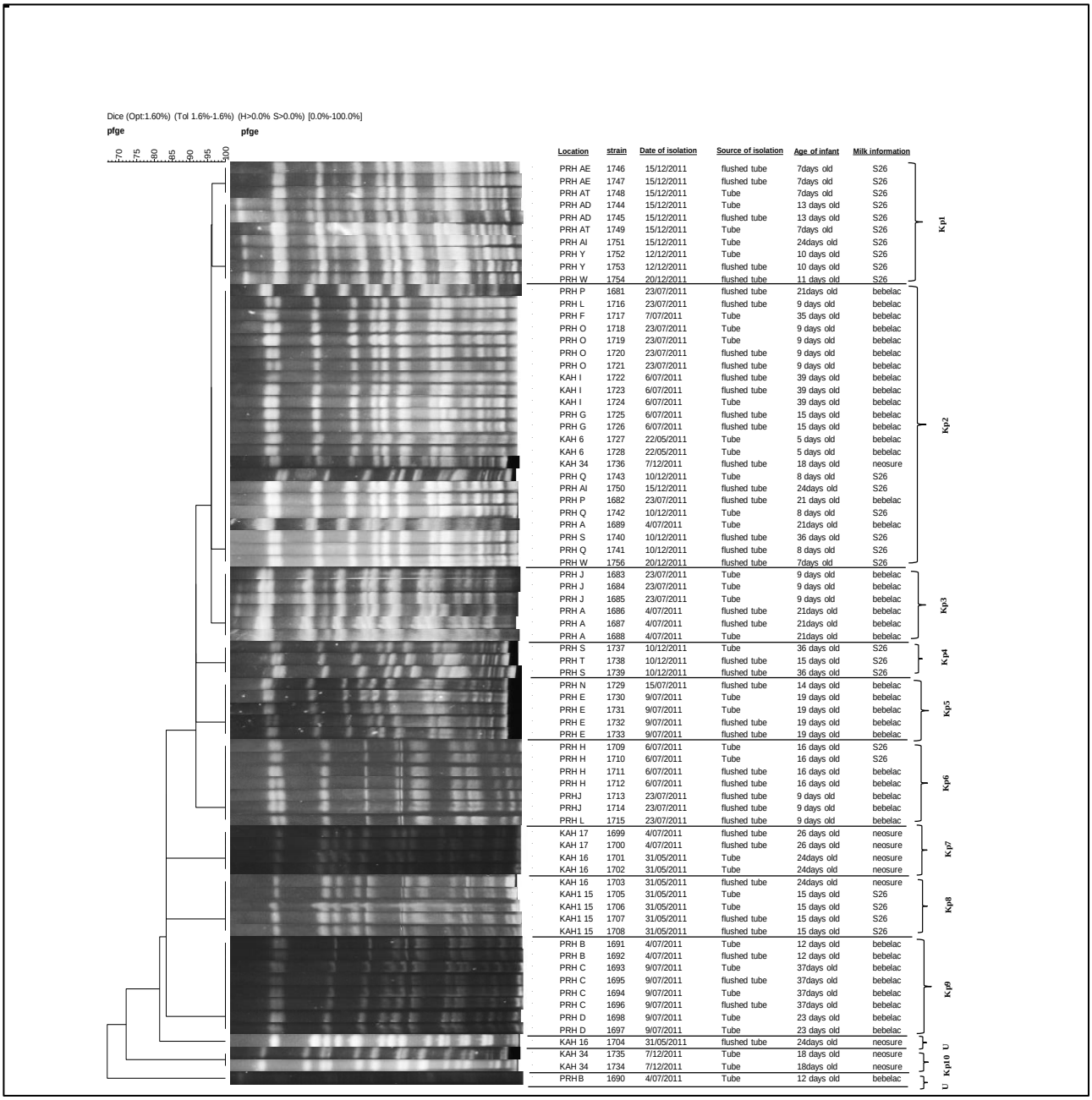
Table 3.5: Summary of PFGE analysis of *Enterobacteriaceae* isolated from mastic human breast milk (MBM).

Species	Strain Number	PFGE cluster	Genetic relatedness
Ent. cancerogenus	1359, 1360	Enc1	closely related
	1355, 1358	Enc2	
	1363, 1364	Enc3	
	1350,1357, 1361, 1362, 1365 & 1367	U	Unrelated
Ent. ludwigii	1348,1349,1351, 1352 & 1366		
K.oxytoca	1353, 1354 & 1356		

Enc1-Enc3 indicate PFGE cluster groups, U: Unique.

Table 3.5 is a summary of Figure 3.5 which presents the analysis of PFGE of twenty strains of *Ent. ludwigii*, *Ent. cancerogenus* and *K. oxytoca* from MBM in Spain. The MBM isolates were clustered into 3 pulsotypes Enc1, Enc2 and Enc3 and 14 unique strains (Figure 3.5, Table 3.5). The PFGE of the MBM isolates revealed that two strains; 1359 and 1360 (Enc1), showed 78% of similarity coefficient while two strains; 1355 and 1358 (Enc2), were 89% similar according to Dice analysis and differed by only 3 bands (Figure 3.5). The similarity coefficient of two strains (1363 and 1364) with the others showed a similarity coefficient of 92%, which were clustered as Enc3. Therefore, they are probably six isolates of the same clone (Tenover *et al*., 1995). Since the original sampling by Delgado was confidential and little information exists with respect to the clustering and typing of strains we do not know if each of the two strains were from the same mother, but this was deemed highly likely. All other strains were < 80% similar and were therefore regarded as unique isolates, i.e. not clonal.

3.3.1.3 Characterisation of *K. pneumoniae* strains isolated from EFT in Jordan



Location	strain	Date of isolation	Source of isolation	Age of infant	Milk information	
PRH AE	1746	15/12/2011	flushed tube	7days old	S26	Kp1
PRH AE	1747	15/12/2011	flushed tube	7days old	S26	
PRH AT	1748	15/12/2011	Tube	7days old	S26	
PRH AD	1744	15/12/2011	Tube	13 days old	S26	
PRH AD	1745	15/12/2011	flushed tube	13 days old	S26	
PRH AT	1749	15/12/2011	Tube	7days old	S26	
PRH AI	1751	15/12/2011	Tube	24days old	S26	
PRH Y	1752	12/12/2011	Tube	10 days old	S26	
PRH Y	1753	12/12/2011	flushed tube	10 days old	S26	
PRH W	1754	20/12/2011	flushed tube	11 days old	S26	
PRH P	1681	23/07/2011	flushed tube	21days old	bebelac	Kp2
PRH L	1716	23/07/2011	flushed tube	9 days old	bebelac	
PRH F	1717	7/07/2011	Tube	35 days old	bebelac	
PRH O	1718	23/07/2011	Tube	9 days old	bebelac	
PRH O	1719	23/07/2011	Tube	9 days old	bebelac	
PRH O	1720	23/07/2011	flushed tube	9 days old	bebelac	
PRH O	1721	23/07/2011	flushed tube	9 days old	bebelac	
KAH I	1722	6/07/2011	flushed tube	39 days old	bebelac	
KAH I	1723	6/07/2011	flushed tube	39 days old	bebelac	
KAH I	1724	6/07/2011	Tube	39 days old	bebelac	
PRH G	1725	6/07/2011	flushed tube	15 days old	bebelac	
PRH G	1726	6/07/2011	flushed tube	15 days old	bebelac	
KAH 6	1727	22/05/2011	Tube	5 days old	bebelac	
KAH 6	1728	22/05/2011	Tube	5 days old	bebelac	
KAH 34	1736	7/12/2011	flushed tube	18 days old	neosure	
PRH Q	1743	10/12/2011	Tube	8 days old	S26	
PRH AI	1750	15/12/2011	flushed tube	24days old	S26	
PRH P	1682	23/07/2011	flushed tube	21 days old	bebelac	
PRH Q	1742	10/12/2011	Tube	8 days old	S26	
PRH A	1689	4/07/2011	Tube	21days old	bebelac	
PRH S	1740	10/12/2011	flushed tube	36 days old	S26	
PRH Q	1741	10/12/2011	flushed tube	8 days old	S26	
PRH W	1756	20/12/2011	flushed tube	7days old	S26	
PRH J	1683	23/07/2011	Tube	9 days old	bebelac	Kp3
PRH J	1684	23/07/2011	Tube	9 days old	bebelac	
PRH J	1685	23/07/2011	Tube	9 days old	bebelac	
PRH A	1686	4/07/2011	flushed tube	21days old	bebelac	
PRH A	1687	4/07/2011	flushed tube	21days old	bebelac	
PRH A	1688	4/07/2011	Tube	21days old	bebelac	
PRH S	1737	10/12/2011	Tube	36 days old	S26	Kp4
PRH T	1738	10/12/2011	flushed tube	15 days old	S26	
PRH S	1739	10/12/2011	flushed tube	36 days old	S26	
PRH N	1729	15/07/2011	flushed tube	14 days old	bebelac	Kp5
PRH E	1730	9/07/2011	Tube	19 days old	bebelac	
PRH E	1731	9/07/2011	Tube	19 days old	bebelac	
PRH E	1732	9/07/2011	flushed tube	19 days old	bebelac	
PRH E	1733	9/07/2011	flushed tube	19 days old	bebelac	
PRH H	1709	6/07/2011	Tube	16 days old	S26	Kp6
PRH H	1710	6/07/2011	Tube	16 days old	S26	
PRH H	1711	6/07/2011	flushed tube	16 days old	bebelac	
PRH H	1712	6/07/2011	flushed tube	16 days old	bebelac	
PRHJ	1713	23/07/2011	flushed tube	9 days old	bebelac	
PRHJ	1714	23/07/2011	flushed tube	9 days old	bebelac	
PRH L	1715	23/07/2011	flushed tube	9 days old	bebelac	
KAH 17	1699	4/07/2011	flushed tube	26 days old	neosure	Kp7
KAH 17	1700	4/07/2011	flushed tube	26 days old	neosure	
KAH 16	1701	31/05/2011	Tube	24days old	neosure	
KAH 16	1702	31/05/2011	Tube	24days old	neosure	
KAH 16	1703	31/05/2011	flushed tube	24days old	neosure	Kp8
KAH1 15	1705	31/05/2011	Tube	15 days old	S26	
KAH1 15	1706	31/05/2011	Tube	15 days old	S26	
KAH1 15	1707	31/05/2011	flushed tube	15 days old	S26	
KAH1 15	1708	31/05/2011	flushed tube	15 days old	S26	
PRH B	1691	4/07/2011	Tube	12 days old	bebelac	Kp9
PRH B	1692	4/07/2011	flushed tube	12 days old	bebelac	
PRH C	1693	9/07/2011	Tube	37days old	bebelac	
PRH C	1695	9/07/2011	flushed tube	37days old	bebelac	
PRH C	1694	9/07/2011	Tube	37days old	bebelac	
PRH C	1696	9/07/2011	flushed tube	37days old	bebelac	
PRH D	1698	9/07/2011	Tube	23 days old	bebelac	
PRH D	1697	9/07/2011	Tube	23 days old	bebelac	
KAH 16	1704	31/05/2011	flushed tube	24days old	neosure	U
KAH 34	1735	7/12/2011	Tube	18 days old	neosure	Kp10
KAH 34	1734	7/12/2011	Tube	18days old	neosure	
PRH B	1690	4/07/2011	Tube	12 days old	bebelac	U

Figure 3.6: PFGE cluster analysis of *K. pneumoniae* strains isolated from EFT in Jordan.

The dendogram was generated by BioNumerics software, version 3.5, dice coefficient and unweight pair group method with arithmetic mean (UPGMA) for cluster analysis. The tolerance and optimisation in the band was 1.60%. Kp1- Kp10 indicates PFGE cluster groups; U: Unique.

Table 3.6: Summary of PFGE analysis of *K. pneumoniae* strains isolated from Jordan hospital.

Species	Hospital	Strain Number	PFGE cluster	Number of Strains	Period of isolation	Patients
K. pneumoniae	PRH	1744, 1745, 1746,1747,1748,1749,1751,1752, 1753,1754	Kp1	10	during 3 days	6
		1681,1682,1689,1716,1717,1718,1719,1720,1721,1722, 1723, 1724, 1725,1726, 1727, 1728, 1736,1740,1741,1742,1743,1750,1756	Kp2	23	over seven months	13
	KAH	1722,1723,1724,1727, 1728				
	PRH	1683, 1684, 1685,1686,1687,1688	Kp3	6	over two weeks	2
		1737, 1738, 1739	Kp4	3	same day	2
		1729, 1730, 1731, 1732, 1733	Kp5	5	one week	2
		1709, 1710, 1711,1712, 1713, 1714,1715	Kp6	7	over two weeks	3
		1691,1692,1693,1694, 1695,1696,1697,1698	Kp9	8	one week	3
		1690	U	1	same day	1
	KAH	1699, 1700, 1701,1702	Kp7	4	over one month	2
		1703,1705,1706,1707, 1708	Kp8	5	same day	2
		1734, 1735	Kp10	2	same day	1
		1704	U	1	one day	1

KAH: King Abdulla Hospital. PRH: Princesses Rahma Hospital. Kp1-Kp10: indicates PFGE cluster groups; U: Unique.

Table 3.6 show the summary of Figure 3.6 which presents the PFGE analysis of Jordan strains. There were seventy five EFT isolates of *K. pneumoniae* from two hospitals in Jordan which were PRH (n=60) and KAH (n=15); [Appendix 7]. The strains were clustered into 4 pulsotypes Kp1–Kp10 and there were two unique strains. The pulsotypes are described briefly below:

A total of ten strains were isolated during a nine day period (12^{th}, 15^{th}, and 20^{th} December 2011). These strains belonged to pulsotype Kp1, and were isolated from 6 different patients receiving one type of infant formula (S26). Ten strains of pulsotype Kp1 were composed of two sub-clusters: containing 3 and 7 strains. The similarity coefficient within each sub-cluster was 100%. Similarity coefficient between the two strains sub-clusters 3 and 7 was 96%.

All strains in pulsotypes Kp2-Kp10 showed 100% of similarity coefficient. Twenty three strains belonged to pulsotype Kp2. This pulsotype was isolated over a prolonged period of five months (4^{th}, 6^{th}, 7^{th} and 23^{rd} July and 10^{th}, 15^{th} and 20^{th} December 2011), from two different hospitals in Jordan. Six strains were isolated from KAH hospital and

seventeen strains were isolated from PRH hospital. These strains were isolated from 13 different neonates where eight patients received bebelac, one received neosure and four received S26 infant formula. The Kp2 pulsotypes strains were isolated from a large number of neonates over a wide time period from two different hospitals. The result appears unlikely and needs further confirmation by using another restriction enzyme.

Six strains of *K. pneumoniae* were grouped into pulsotype Kp3. These strains were isolated from two different neonates over two weeks (4th and 23rd July 2011). The hospitals used bebelac for infant feed.

Three strains of *K. pneumoniae* belonged to pulsotype Kp4, which were isolated on the same day (10th December 2011) and the strains were isolated from two different neonates, receiving S26 infant formula.

Pulsotype Kp5 was composed of five strains of *K. pneumoniae*. There were isolated on one week (9th and 15th July 2011) from 2 patients, receiving bebelac infant formula.

Seven strains of *K. pneumoniae* formed pulsotype Kp6, which were isolated over a two week period (6th and 23rd July 2011) from 3 different patients receiving S26 and bebelac infant formula.

From 2 different patients, 4 isolates of *K. pneumoniae* were recovered which belonged to pulsotype Kp7. These strains were isolated over one months (31st May and 4th July 2011).The babies were received neosure infant formula.

Five strains of *K. pneumoniae* belonged to pulsotype Kp8, which were isolated on the same day (31st May 2011) from two different patients, receiving different types of infant formula (S26 and bebelac).

At one week period (4th and 9th July 2011) eight strains of *K. pneumoniae* were isolated which belonged to pulsotype Kp9. These were isolated from 3 different patients, receiving bebelac infant formula.

Two strains of *K. pneumoniae* belonged to pulsotype Kp10, which were isolated on the same day (7th December 2011) and from the same patient receiving neosure infant formula.

Two strains (1704 and 1690) were unique and were isolated from two different patients receiving neosure and bebelac infant formula respectively.

3.3.2 Comparison of phenotypic and genotypic techniques for identification of *Enterobacteriaceae*

All strains were identified using biochemical profiles based on API 20E and ID32E methods. Genotyping identification was also used to identify the isolates by using 16S rDNA sequence analysis. In addition, the phenotypic and genotypic results were compared with PFGE results.

3.3.2.1 Identification of *Enterobacteriaceae* strains isolated from EFT

Table 3.7 part1 shows the comparison between phenotyping and genotyping identification technique. Most *Ent. cloacae* strains were re-identified by 16S rDNA sequence analysis and were subsequently re-assigned as *Ent. hormaechei*. There were clustered into 7 clusters Ebh1-Ebh7 and four unique strains by using PFGE technique.

An interesting result was that two strains which were initially identified as *K. oxytoca* by ID32E, but were re-identified by 16S rDNA sequence analysis as *Ent. aerogenes*. There were clustered in one cluster Eba1. Another two strains were identified by ID32E tests as *Ent. cloacae* and *Ent. amnigenus* however, were re-identified by 16S rDNA sequence analysis as *Ent. hormaechei*. They were clustered in one cluster Ebh7.

In Table 3.7 part 2 shows that fifty one strains were previously identified as *Klebsiella* spp. and *E. coli*; six strains were identified as *K. pneumoniae* and 44 strains were identified as *K. oxytoca*, whereas one strain was *E. coli* by using API20E/ID32E tests. While in this study these strains were re-identified by 16S rDNA sequence analysis as *Ent. cancerogenus*, but their pulsotypes clustered into 6 clusters Ebc1-Ebc6 and two unique strains.

All *K. pneumoniae* and 15 of *K. oxytoca* strains belonged to one cluster; pulsotype Ebc2. On the other hand, strains 1037 and 1088 were identified as *K. oxytoca* and *E. coli* respectively by ID32E, but they were identified as *Ent. cancerogenus* by 16S rDNA gene sequence analysis and both belonged to cluster Ebc6.

All strains in table 3.8 were identified as *Klebsiella* spp. by both API20E/ID32E tests and were in 6 pulsotypes Kc1-Kc6 and one unique strain. The exception was in clusters Kc1 and Kc6 which consisted of only one *E. coli* and 3 *Ent. aerogenes* strains respectively, as identified by ID32E test. However when re-identified by 16S rDNA, the strains in both Kc1 and Kc6 clusters were found to be *K. pneumoniae*.

Table 3.7: Identification *Enterobacteriaceae* isolated from enteral feeding tubes by using phenotyping and genotyping techniques; part 1.

Strain no.	Source	ID profile	ID API	16S rDNA sequence	PFGE cluster
979	NCH	3305573[a]	*Ent. cloacae*	*Ent.hormaechei*	Ebh1
980		3305573[a]	,,	,,	
981		3305573[a]	,,	,,	
983		3305573[a]	,,	,,	
986		3305573[a]	,,	,,	
987		3305573[a]	,,	,,	
993		3305573[a]	,,	,,	
997		3305573[a]	,,	,,	
856		7305573[a]	,,	,,	Ebh4
859		7305573[a]	,,	,,	
860		7305573[a]	,,	,,	
861		7305573[a]	,,	,,	
862		7305573[a]	,,	,,	
863		7305573[a]	,,	,,	
795		7305573[a]	,,	,,	Ebh6
797		7305573[a]	,,	,,	
798		7305573[a]	,,	,,	
799		7305573[a]	,,	,,	
800		7305573[a]	,,	,,	
801		7305573[a]	,,	,,	
802		7305573[a]	,,	,,	
803		7305573[a]	,,	,,	
960		3305573[a]	,,	,,	U
790		7305573[a]	,,	,,	
779		7244773[a]	*K. oxytoca*	*Ent. cloacae*	Ebl1
789		7244773[a]	*K. pneumoniae*	,,	
1033	QMC	73015732[a]	*Ent. cloacae*	*Ent.hormaechei*	Ebh2
1034		73015732[a]	,,	,,	
1035		73015732[a]	,,	,,	
1052		34064753331[b]	,,	,,	
1074		74064753331[b]	,,	,,	
1032		73015732[a]	,,	,,	Ebh3
1053		34064753331[b]	,,	,,	
1075		34064753331[b]	,,	,,	
1027		73015632[a]	,,	,,	Ebh5
1066		34064751131[b]	,,	,,	
1067		34064751131[b]	,,	,,	
1081		34064751131[b]	,,	,,	
1084		34064751131[b]	,,	,,	
1068		34064751131[b]	,,	,,	
1069		34064751131[b]	,,	,,	
1089		34064751131[b]	,,	,,	
1044		34060743231[b]	,,	,,	Ebh7
1038		34074743231[b]	*Ent. amnigenus*	,,	
1039		34060743231[b]	*Ent. cloacae*	,,	
1040		34060743231[b]	,,	,,	
1058		47175757333[b]	*K. oxytoca*	*Ent. aerogenes*	Eba1
1056		44175757333[b]	,,	,,	
1028		73055733[a]	*Ent. cloacae*	*Ent. cloacae*	U

QMC: Queen's Medical Centre, NCH: Nottingham City Hospital. a and b indicate type of phenotyping test API 20E and ID32E respectively. Ebh1- Ebh7, Ebl1 and Eba1 indicate PFGE cluster groups, U: Unique.

Table 3.7: Identification *Enterobacteriaceae* isolated from enteral feeding tubes by using phenotyping and genotyping techniques; part 2.

Strain no.	Source	ID profile	ID API	16S rDNA sequence	PFGE cluster
982	NCH	7245773[a]	*K. oxytoca*	*Ent. cancerogenus*	Ebc1
985		7245773[a]	,,	,,	
988		7245773[a]	,,	,,	
989		7245773[a]	,,	,,	
992		7245773[a]	,,	,,	
994		7245773[a]	,,	,,	
996		7245773[a]	,,	,,	
998		7245773[a]	,,	,,	
990		7245773[a]	,,	,,	
991		7245773[a]	,,	,,	
845		7245573[a]	,,	,,	
847		7245573[a]	,,	,,	
848		7245573[a]	,,	,,	
850		7245573[a]	,,	,,	
851		7245573[a]	,,	,,	
907		7245773[a]	,,	,,	Ebc2
908		7245773[a]	,,	,,	
909		7045773[a]	,,	,,	
782		7244773[a]	,,	,,	Ebc3
783		7244773[a]	,,	,,	
781		7244773[a]	,,	,,	
784		7204773[a]	*K. pneumoniae*	,,	
807		7244773[a]	*K. oxytoca*	,,	
808		7244773[a]	,,	,,	
810		7245773[a]	,,	,,	
814		7245773[a]	*K. pneumoniae*	,,	
815		7245773[a]	,,	,,	
816		7245773[a]	,,	,,	
817		7245773[a]	,,	,,	
818		7245773[a]	,,	,,	
824		7245773[a]	*K. oxytoca*	,,	
825		7245773[a]	,,	,,	
826		7245773[a]	,,	,,	
827		7245773[a]	,,	,,	
828		7245773[a]	,,	,,	
829		7245773[a]	,,	,,	
831		7245773[a]	,,	,,	
834		7245773[a]	,,	,,	
842		7245773[a]	,,	,,	
957		7245773[a]	,,	,,	Ebc5
966		7245773[a]	,,	,,	
969		7245773[a]	,,	,,	
972		7245773[a]	,,	,,	
806		7244773[a]	,,	,,	U
1077	QMC	47575777333[b]	*K. oxytoca*	*Ent. cancerogenus*	Ebc4
1042		47175757233[b]	,,	,,	
1054		47075377333[b]	,,	,,	
1059		44175757333[b]	,,	,,	
1071		47175057333[b]	,,	,,	
1037		45174757333[b]	,,	,,	Ebc6
1088		75565763420[b]	*E. coli*	,,	

QMC: Queen's Medical Centre, NCH: Nottingham City Hospital. a and b indicate type of API test ID20E and ID32E respectively. Ebc1- Ebc6 indicates PFGE cluster groups, U: Unique.

Table 3.8: Identification *Enterobacteriaceae* isolated from enteral feeding tubes by using phenotyping and genotyping techniques.

Strain no.	Source	ID profile	ID API	16S rDNA sequence	PFGE cluster
778	NCH	7214773[a]	*K. pneumoniae*	*K. pneumoniae*	Kc2
785		7205773[a]	"	"	
787		7205773[a]	"	"	
788		7205773[a]	"	"	
792		7205773[a]	"	"	
794		7205773[a]	"	"	
832		7255773[a]	*K. oxytoca*	*K. oxytoca*	Kc5
833		7255773[a]	"	"	
830		7254773[a]	"	"	
839		7215773[a]	*K. pneumoniae*	*K. pneumoniae*	Kc4
841		7215773[a]	"	"	
976		7215773[a]	"	"	Kc6
977		7215773[a]	"	"	
999		7215773[a]	"	"	
1004		7215773[a]	"	"	
1006		7215773[a]	"	"	
1000		7215773[a]	"	"	
1002		7215773[a]	"	"	
1005		7215773[a]	"	"	
1001		7215773[a]	"	"	
1003		7215773[a]	"	"	
1012		7315773[a]	*Ent. aerogenes*	"	
1013		7315773[a]	"	"	
1014		7315773[a]	"	"	
1090	QMC	45174757231[b]	*K. pneumoniae*	*K. pneumoniae*	Kc1
1091		45174757231[b]	"	"	
1092		45170757231[b]	"	"	
1046		45174757231[b]	"	"	
1048		44465543420[b]	*E.coli*	"	
1049		45174757231[b]	*K. pneumoniae*	"	
1078		46575777333[b]	*K. oxytoca*	*K. oxytoca*	Kc3
1079		46575777333[b]	"	"	
1093		45074757331[b]	*K. pneumoniae*	*K. pneumoniae*	U

QMC: Queen's Medical Centre, NCH: Nottingham City Hospital. a and b indicate type of phenotyping test API 20E and ID32E respectively. Kc1- Kc6 indicates PFGE cluster groups, U: Unique.

3.3.2.2 Identification of *Enterobacteriaceae* isolated from mastic human breast milk

The strains previously isolated from MBM were further analysed by phenotyping using ID32E following the procedure in section 2.2.1. The results are summarised in Table 3.9. As shown, all strains were identified as *K. oxytoca*. 16S rDNA gene sequence analysis was used to identify the recovered bacterial strains. As shown in Table 3.9, there was variation between ID32E results and 16S identification. The results obtained for the MBM isolates showed that out of the twenty strains identified as *K. oxytoca* by ID32E, twelve were *Ent. cancerogenus* and five were *Ent. ludwigii* with the more reliable 16S rDNA sequence analysis; see Table 3.9.

The interesting finding was that six strains which were *Ent. cancerogenus* (1359, 1360, 1355, 1358, 1363 and 1364) were identified as *K. oxytoca* by ID32E test and their ID profile was different; Table 3.9. However, these strains were identified by 16S rDNA sequence analysis as *Ent. cancerogenus* and their pulsotypes were Enc1, Enc2 and Enc3. Each of the two strains belonged to one cluster respectively; see Table 3.9.

Table 3.9: Identification *Enterobacteriaceae* isolated from mastic human breast milk by using phenotyping and genotyping techniques.

Strain no.	ID32E profile	ID32E	16S rDNA sequence	PFGE cluster
1350	4 7 1 7 5 7 5 7 3 3 1	*K. oxytoca*	*Ent. cancerogenus*	U
1355	4 7 1 7 5 7 5 7 3 3 1	„	„	Enc2
1358	5 7 1 7 5 7 5 7 3 3 1	„	„	
1357	4 7 1 7 5 7 5 7 3 3 1	„	„	U
1359	4 7 5 7 5 7 5 7 3 3 1	„	„	Enc1
1360	4 7 1 7 5 7 5 7 3 3 0	„	„	
1361	4 7 5 7 5 7 5 7 1 3 1	„	„	U
1362	4 7 5 7 5 7 5 7 3 3 1	„	„	
1363	6 7 1 7 5 7 5 7 3 3 1	„	„	Enc3
1364	7 7 1 7 5 7 5 7 3 3 1	„	„	
1365	7 7 1 7 5 7 5 7 3 3 1	„	„	
1367	4 7 1 7 5 7 5 7 3 3 1	„	„	
1348	4 7 0 7 5 7 7 7 3 7 3	„	*Ent. ludwigii*	
1349	4 7 5 7 5 7 5 7 3 3 1	„	„	
1351	4 7 5 7 5 7 5 7 3 3 1	„	„	U
1352	4 7 1 7 5 7 5 7 3 3 0	„	„	
1366	4 7 1 7 4 7 1 7 3 3 1	„	„	
1353	4 7 5 7 5 7 5 7 3 3 1	„	*K. oxytoca*	
1354	6 5 1 7 5 7 5 7 3 3 1	„	„	
1356	4 4 1 7 5 7 5 7 3 3 1	„	„	

Enc1- Enc3 indicates PFGE cluster groups, U: Unique.

3.4 DISCUSSION

This project investigated the neonatal of infection with *Enterobacteriaceae* from MBM from Spain and EFT using strains from QMC and NCH (UK) and Jordan. The aim of this investigation was to assess neonatal exposure to bacteria. These studied strains do not have any associated clinical details; nevertheless, the aim of assessing and characterising strains from these three sources is important. EFT isolates of *K. pneumoniae* (from Jordan) were also analysed but only for PFGE profiling due to time constraints. Strains isolated from MBM and EFT included bacteria which are potentially pathogenic to neonates. The present study is a continuation the studies of Hurrell *et al.* (2009b) and Delgado *et al*. (2008) and has further investigated and characterised their strains. PFGE was applied to the EFT from QMC and NCH (UK), EFT isolates of *K. pneumoniae* (from Jordan) and MBM (Spain).

In this study, one hundred and thirty-three *Enterobacteriaceae* strains have been obtained from two local hospitals in Nottingham, NCH and QMC. These strains were isolated from neonatal enteral feeding tubes; forty four of *Ent. hormaechei*, fifty one of *Ent. cancerogenus*, thirty three of *Klebsiella* spp., three strains of *Ent. cloacae* and two strains of *Ent. aerogenes*. These strains were isolated from 60 patients during 10 months and the period was from 17th January to 19th November 2007. This study applied PFGE as a DNA fingerprinting technique to identify the source of isolation, determine if the same strains were isolated at different times from the NICU and to confirm whether these strains caused the contamination of EFT which in turn increased the risk of exposure to neonates.

The results of this investigation showed that the *Ent. hormaechei* strains were clustered into 7 pulsotypes (Ehc1-Ehc7) in addition to the two unique strains identified (Figure 3.1, Table 3.1). *Ent. cancerogenus* strains were clustered together into 6 pulsotypes (Ebc1–Ebc6) and two unique strains; (Figure 3.2, Table 3.2). *Klebsiella* spp. were grouped into 6 pulsotypes and one unique strain (Figure 3.3, Table 3.3). The last group consisted of multiple isolates, *Ent. cloacae* and *Ent. aerogenes* which were clustered into two pulsotypes (Eba1 and Ebl1) and one unique strain. Each Eba1 and Ebl1 comprised of 2 isolates; (Figure 3.4, Table 3.4).

The majority of the pulsotypes were isolated from the same patient in one day. For example, eight strains of *Ent. hormaechei* and six strains of *K. pneumoniae* (Figure 3.1, Table 3.1) were isolated from the same patient on 16th February 2007 and 30th January 2007 respectively. These strains were clustered in Ebh1 and Kc2 (Figures 3.1 and 3.3). It is possible that the strain multiplied and contaminated the feeding tubes in a short time. The most explicable finding in this study is the identification of clusters Ebh6 and Kc2. The cluster Ebh6 comprised of eight strains which were isolated from six patients on the same day, while cluster Kc2 consisted of six strains isolated on the same day from six different patients. The results of this study indicate that one clone of each cluster potentially cross contaminated the neonatal enteral feeding tubes with diverse pulsotypes of *Ent. hormaechei* and *K. pneumoniae* during one week (23rd and 30th January 2007).

One of the significant findings in this study is that three different clusters were isolated over a wide time period. There were eight strains of *Ent. hormaechei* in pulsotype Ebh5 which were isolated from 3 patients in one month (16th October and 12th and 19th November 2007), twenty one strains of *Ent. cancerogenus* pulsotypes Ebc3 were isolated from 13 patients over two months (16th, 17 th and 23rd January, 6th,11th,13th,16th, 17th and 20th February and 20th March 2007) and 6 strains of *K. pneumoniae* pulsotype Kc1 were isolated from two patients in one month (22nd October and 19th November 2007). Taken together, these results suggest the cross-transmission of indistinguishable clones of each pulsotype between different patients over a long period of time in the same NICUs. This finding may indicate the same origins, such as: environment, infant formula or carers.

Furthermore, the collection of seventy five *K. pneumoniae* strains isolated from the EFT of neonates in NICUs from two hospitals in Jordan were analysed by using PFGE. The objective was to detect strains that were isolated at different times from the NICUs and to determine whether the strains have colonised the neonatal EFT which might have caused the colonisation in the NICUs leading to potentially increased exposure and risk to the neonates.

The PFGE result of Jordan cultures indicated genetically indistinguishable *K. pneumoniae* strains, forming two small pulsotype clusters, Kp1 and Kp4, containing

ten and two strains respectively. It also showed that large groups Kp2 and Kp3 were comprised of forty and twenty three strains respectively.

The Kp2 pulsotype was isolated over a prolonged period of seven months (22nd May, 4th, 7th, 23rd July, 7th, 10th, 15th and 20th December 2011) from two different hospitals; KAH and RAH. A total of 23 strains belonged to Kp2 cluster isolated from 13 different patients; six of the strains were isolated from KAH hospital, while seventeen strains were from RAH hospital.

Similarly, Kp3 pulsotype had formed one large pulsotype group which included 6 strains. This cluster was isolated over two weeks period (4th and 23rd July 2011) from 6 patients and the babies were fed the same type of infant formula, bebelac.

In addition, Kp2 and Kp3 were isolated from thirteen and two patients respectively and bebelac infant formula was used to feed the babies. This finding indicates the existence of a clone for each group which showed 100% of similarity coefficient. Furthermore, the cross-transmission of an indistinguishable clone was determined from different neonates over a long period.

Of particular interest was the Kp2 pulsotype; the result is quite unlikely since the strains were isolated from different of two hospitals. However, the most likely explanation seems to be the likelihood that both of these hospitals in Jordan used the same brand of infant formula “bebelac” which could have been contaminated with bacterial strains in Kp2 cluster. The KAH hospital fed the babies with bebelac in May and the PRH hospital started using this brand in July. So this clone possibly cross contaminated the neonatal enteral feeding tubes over a wide time period (from May to December) and infected a large number of neonates. However, this assumption needs verification of these results using a different restriction enzyme in the PFGE.

In this study, an interesting finding is the indistinguishable clone of *K. pneumoniae* Kp2 and Kp3 clusters contaminating the feeding tubes in two different hospitals which could have originated from the same source. The possible sources could be the powdered infant formula, preparation of PIF for infants in NICU or the hands of care workers. This

can result in the contamination of fresh feed in the tube lumen leading to further bacterial multiplication.

In this study, the findings of results for two sources of EFT from QMC and NCH (UK) and EFT from Jordan were consistent with FAO/WHO (2004, 2006) which found the PIF is not a sterile product and maybe an important source of bacterial pathogens that can cause serious illness in infants (i.e., children aged <1 year). In addition, previous studies by Muytjens *et al*. (1988) and Townsend *et al.* (2008a) reported that *Enterobacteriaceae* isolated from PIF include *Ent. cloacae*, *K. pneumoniae*, *K. oxytoca*, *Ent. hormaechei*, *Cit. freundii*, and *E. coli*. Furthermore, studies by Caubilla-Barron *et al.* (2007) and Iversen *et al*. (2008) found that *C. sakazakii* and *Salmonella* were associated with contaminated PIF which caused neonatal infections.

The reconstitution temperature of PIF for infants in NICUs and the storage period could increase the growth of any bacteria and cross contamination in NICUs is then very likely. Forsythe (2009) reported that in a neonatal care unit, PIF (fortified breast milk and reconstituted powdered infant formula) was reconstituted at room temperature when required, not at higher temperatures. This procedure in NICUs across the UK may differ.

A further research study in French hospitals by Rosset *et al*. (2007) reported that similar practices were used. They stated that samples were prepared with cold water (room temperature), kept at room temperature for <15 minutes and then stored in a cold cabinet at 4°C or below. In a water bath, multiple bottle heating was used to reheat samples for 30 minutes. At the end of reheating, the temperatures were less than 47°C for 55% of the samples. The recommendation by FAO/WHO in 2006 is to limit the time from preparation to consumption and also prepared feeds should be stored at temperatures not more than 5°C.

Another potential source of bacteria could be through a contaminated healthcare worker, which could lead to bacterial transmission between the patients. This may be consistent with previous studies that report the source of a nosocomial outbreak of colonisation and infection with a strain of *Se. marcescens* was from health care workers. This occurred in

an ICU in a Dutch University Medical Center from May 2002 through March 2003 (de Vries *et al.*, 2006).

The healthcare workers suffering from chronic otitis external have been linked epidemiologically with the outbreak of Methicillin-Resistant *Staphylococcus aureus* colonisation and infection in a NICU (Bertin *et al*., 2006). A study by Waters *et al*. (2004) detected Gram-negative bacilli previously associated with infections in neonates on the nurses' hands, even after the hand hygiene was performed.

Out of the twenty strains isolated from MBM, six strains of *Ent. cancerogenus* were divided into three clusters; Enc1- Enc3. The interesting finding is strains 1355 and 1358 (Enc2), were found to have 89% similarity patterns (Figure 3.5), and two strains 1359 and 1360 (Enc1) showed 78% of similarity coefficient, while cluster Enc3 (1363 and 1364) showed a similarity coefficient of 92%. These six strains were also found to be nearly identical by using 16S rDNA sequence analysis; additionally, the strains differ by only 3 bands by PFGE (Tenover *et al.*, 1995). All other strains showed pattern < 80% similar patterns and, therefore, were regarded as unique isolates, i.e. not clonal. The results for the MBM isolates showed six strains were of particular interest which belonged to three pulsotypes. They were probably the same clone and most likely obtained from the same mother.

Two strains, 779 and 789, were isolated from EFT (QMC and NCH) which were then identified using phenotypic and genotypic techniques. The phenotypic methods, including API20E and ID32E, identified the strains as *Ent. cloacae* and *K. pneumoniae*, while the genotypic method 16S identified both strains as *Ent. cloacae*. PFGE clustered both of these strains in Ebl1. This confirms that the 16S identification is more reliable than the phenotypic methods.

Another important finding is that (Table 3.7 parts 1 and 2) there were two clusters, Kc1 and Kc6, which included one *E. coli* and three strains of *Ent. aerogenes* respectively. These strains were initially identified by the ID32E test, but by 16S rDNA sequence analysis, they were re-assigned as *K. pneumoniae* in both clusters.

Also strains from MBM were characterised using standard phenotyping methods and compared with genotyping. These included *Ent. cancerogenus*, which previously had been misidentified as *K. oxytoca* by using the more common biochemical identification kits, compared with 16S sequence analysis (Table 3.9).

It is of particular interest that six strains of *Ent. cancerogenus* were clustered into three pulsotypes (Ecn1- Ecn3) and were misidentified as *K. oxytoca* by ID32E. This finding indicted that the phenotypic method incorrectly identified most of the strains compared with the genotypic technique. This finding was confirmed with PFGE results which showed similarity classification. The identities of these strains are presented in Table 3.7 parts 1, 2 and 3.8 and Table 3.9.

The most probable explanation is that genotypic identification is more accurate than phenotyping and characterisation of bacteria by PFGE is a suitable technique for strain relatedness.

The findings of the current study were consistent with Iversen *et al*. (2004b and 2004c), who reported that the API 20E and ID32E tests are imperfect techniques to utilise for identification of bacteria because they could give false-negative and false-positive results. Moreover, 16S rDNA sequence analysis results in the current study have agreed with the findings of Iversen *et al*. (2004b and 2006b), who demonstrated that some strains identified as *C. sakazakii* by a biochemical test belonged to diverse species, including two species *Ent. amnigenus* and *Ent. cloacae*.

Furthermore, Townsend *et al*. (2008a) acquired '*Cronobacter*' (*C. sakazakii*) strains from a hospital for further analysis which were identified by using the phenotypic technique. Townsend *et al*. (2008a), using PFGE, revealed that they were clonal and were isolated from a NICU outbreak which the hospital was not aware of. Subsequent 16S rDNA sequencing re-identified the strains as *Ent. hormaechei*. Hence, the hospital had not only not recognised they had an outbreak but had also misidentified the organisms by using incorrect techniques.

Additionally, Muytjens *et al.* (1988) highlighted the incidence of *Cronobacter* spp. (*C. sakazakii*) in powdered infant formula. Initially these strains were identified in 1988

using biochemical profiling, whereas Townsend *et al.* (2008a) have used 16S rDNA sequence analysis to re-identify the strains, some of which were *Ent. hormaechei*. This indicated that Muytjens *et al.* (1988) had misidentified the organisms which were present in powdered formula that could be a source of exposure to neonates because of using unsuitable identification methods.

In summary, neonatal enteral feeding tubes and mastic human breast milk were contaminated by a total of twenty-four pulsotypes of *Enterobacteriaceae*; 21 EFT and 3 MBM. The same pulsotypes spread among the EFT of infants in the same NICUs, indicating the same origins, such as: environment, infant formula or carers. Genotypic identification is a more reliable technique to identify strains than phenotyping and characterisation of bacteria by PFGE is a suitable technique for strains relatedness.

Chapter 4: PHYSIOLOGICAL TRAITS OF BACTERIAL ISOLATES

4.1 Introduction

Enteral feeding tubes are widely used to feed low birth weight infants in NICUs and may be *in situ* for several days. A previous study demonstrated that the enteral feeding tube may be a very important source of bacteria entering neonates, and could also be a place for opportunistic intestinal pathogens to multiply (Hurrell *et al.*, 2009b). *Enterobacteriaceae* are able to form biofilms on enteral feeding tubes as they can attach to inert surfaces and grow (Murga *et al.*, 2001; Thompson *et al.*, 2006; Zogaj *et al.*, 2003).

Lately, considerable attention has been focused on the microbiological safety of PIF. The FAO/WHO (2004) carried out a risk assessment of the organisms associated with neonatal infections and PIF. They categorised the organisms into groups: Category 1 as 'clear evidence of causality', which were *Cronobacter* spp. (formely *Enterobacter sakazakii*) and *Salmonella* serovars, while other *Enterobacteriaceae* were considered Category 2 'causality plausible'.

In 2006 FAO/WHO added *Acinetobacter* spp. to the latter group. These organisms are opportunistic pathogens, although at present there is no evidence of any outbreaks in NICUs of these organisms. NTU, in collaboration with local NICUs, discovered that the organism was present in EFT from neonates on non-formula feeding regimes (Hurrell *et al.*, 2009b). This indicates that neonate exposure to *Cronobacter* may not exclusively be due to reconstituted formula, and the increased exposure to bacterial pathogens may be linked to more general feeding practices.

From two local NICUs in Nottingham, the bacteria from 129 neonatal enteral feeding tubes were studied by Hurrell *et al.* (2009a and b). According to their study, *Enterobacteriaceae* due to their ability to adhere and multiply on feeding tubes, posed a far greater risk to neonatal health than *Cronobacter* alone. This was evident from the isolation of a wide range of *Enterobacteriaceae* from 76% of the feeding tubes which included *E. coli, Ent. cancerogenus, Ent. hormaechei, K. pneumoniae, K. oxytoca, Raoutella* spp., *Se. liquefaciens* and *Se. marcescens,* in addition to *Cronobacter* and *Yersinia* spp.

The infants fed reconstituted PIF showed the highest amount of *Enterobacteriaceae* biofilm formation averaging 4.2 $\log_{10}$ cfu/tube. Longer duration (48 hours) of milk in the feeding tube and the absence of antibacterial agents, unlike breast milk, may allow these bacteria to multiply and grow. Compared to the tubes of infants fed fortified breast milk (3.6 $\log_{10}$ cfu/tube), the lowest number of bacteria was obtained from the EFT of infants fed unfortified breast milk (1.4 $\log_{10}$ cfu/tube).

Biofilms of *Enterobacteraceae* were recovered from 81% of neonates fed sterile ready to feed formula. It was proposed that the gastroesophageal reflux from the throat could be the source of bacteria. The study by Hurrell *et al.* (2009a) also indicated the ability of bacteria to grow in the nasogastric tubes of 10^7cfu/tube within 8 hours which increased to 10^9 cfu/tube within 24 hours, *in situ.* It is plausible that the bacterial biofilm *in vivo* may contaminate the routine feeds and on ageing may detach and survive the passage through the neonate's stomach ultimately reaching the intestial as a bolus with the feed. Therefore, the biofilm formation on the EFT pose a great risk to the neonate's health and should be considered as an important risk factor.

It was concluded from this study that it was important to focus on the microbiological safety of neonatal feed, not only with respect to *Cronobacter* spp. and infant formula but that attention should also be given to the general practices of the feed preparation (Holý and Forsythe, 2014). Subsequently, this may reduce the neonate's exposure to a wide variety of *Enterobacteraceae,* some of which could be resistant to antibiotics. Similarly, the placement of feeding tube in neonates for prolonged durations should also be considered to reduce the exposure to bacterial pathogens. Thus, the biofilm formation on the EFT represents a significant risk factor for neonatal infections.

Acidity of stomach acts as an important line of defence in protecting against infections through ingestion (Martinsen *et al.*, 2005; Smith, 2003). According to Hurrell *et al*. (2009b) reported that the gastric stomach's pH value of neonates fed breast milk, ready-to-feed and powdered infant formulas ranges from 2.5, 3.5 and 4.3, respectively. The several of bacteria are killed at a pH less than 2.0 (Zhu *et al*., 2006). The bacterial killing in the stomach is also simplified by the proteolytic activity of the gastric enzymes as they

cause the release of peptides from, for instance, lactoferrin play important role as antimicrobials (Nibbering *et al*., 2001; Ryley, 2001).

The aims of this chapter was to determine the pertinent physiological traits of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cancerogenus*, *Ent. cloacae Ent. aerogenes* and *K. oxytoca*. Biofilm formation (Stepanovic *et al.*, 2004) on plastic surfaces was determined using casein, whey and soy- based infant formulas at 20°C and 37°C. Previous studies on *Cronobacter* (NTU, unpublished) have shown that biofilm formation is affected by formula type and temperature, such that more attachment occurs at body temperature and, therefore, when the organism comes in contact with the enteral feeding tube *in situ*. Biofilm formation analysed using the cell wall structures could be adherence to surfaces; such as capsule. Heat tolerance to 55°C was used as a representative temperature used for the reconstitution of powdered infant formulas. The survival curve of strains at pH 3.5 (HCl acidified formula) was determined as this is a representative of the neonatal stomach conditions on ingestion (Hurrell *et al.*, 2009b). The collation of this information is useful in identifying the potential exposure of neonates to strains due to heat tolerance, biofilm formation and acidic pH survival as potential risks to neonates in NICUs.

4.2 Materials and methods

The detailed preparation of culture media and experimental conditions have been described previously in section 2.4.

Selected strains of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cancerogenus, Ent. cloacae*, *Ent. aerogenes* and *K. oxytoca* were used in this chapter. Their sources and designated culture collection numbers are preceded in Tables 2.7 (chapter 2). Three types of formula, whey (Cow and Gate), casein (SMA1), and soy-based (Cow and Gate infasoy) infant formula were used to grow bacteria cultures for 18-20 hours (stationary phase) at different temperatures depending on the experimental protocol. The procedure for this chapter has been described earlier:

- Heat tolerance in section 2.4.1
- Biofilm formation in section 2.4.2
- Capsule formation in section 2.4.3

- Acid tolerance in section 2.4.4

Pathogenic bacteria in PIF should not be considered safe at any level because illnesses resulting from the management of contaminated PIF do not need a high number of pathogens, as even small numbers can colonize the neonate and cause disease. However, in this study, the heat tolerance of the isolate was carried out by D-value determination which was calculated as the time required for a 1 log reduction in the viable count at 55ºC. The results were then classified as following:

1- The D- value of less than 4 minutes indicates that the strains are heat sensitive.
2- The D-value between 4 minutes to 15 minutes indicates that the strains are moderatley tolerant to heat.
3- The D- value of more than 15 minutes indicate that the strains are heat resistant.

In the UK, to prepare PIF it sould use water that has been boiled in a kettle and left to cool for 30 minutes, These products are not reconstituted with water and cannot be exposed to heat treatment to kill intrinsic bacteria. Thus, the time required to kill the bacteria less than 15 minutes is still milk hot to fed neonates.

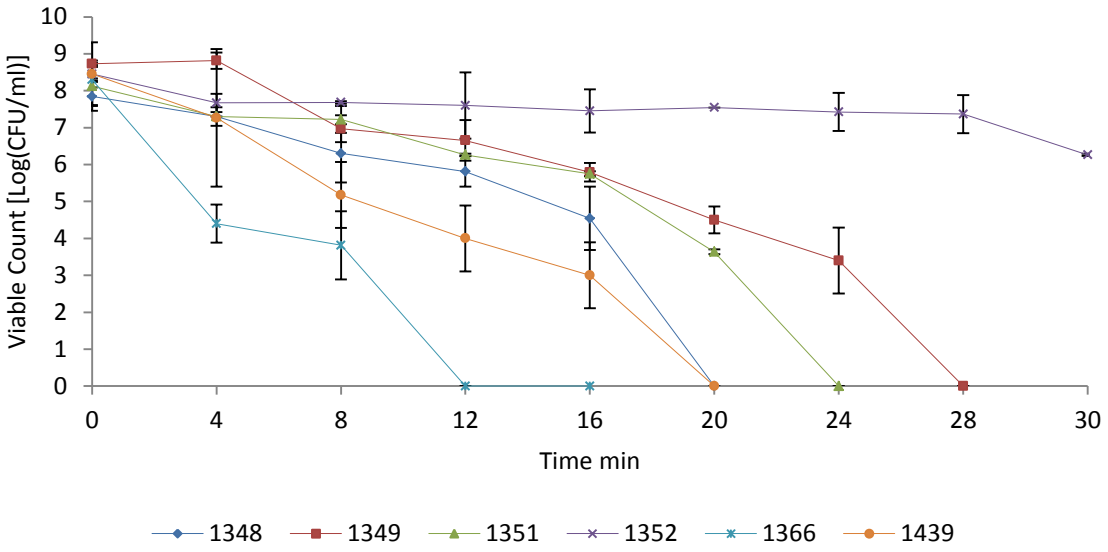
4.3 Results

4.3.1 Heat tolerance

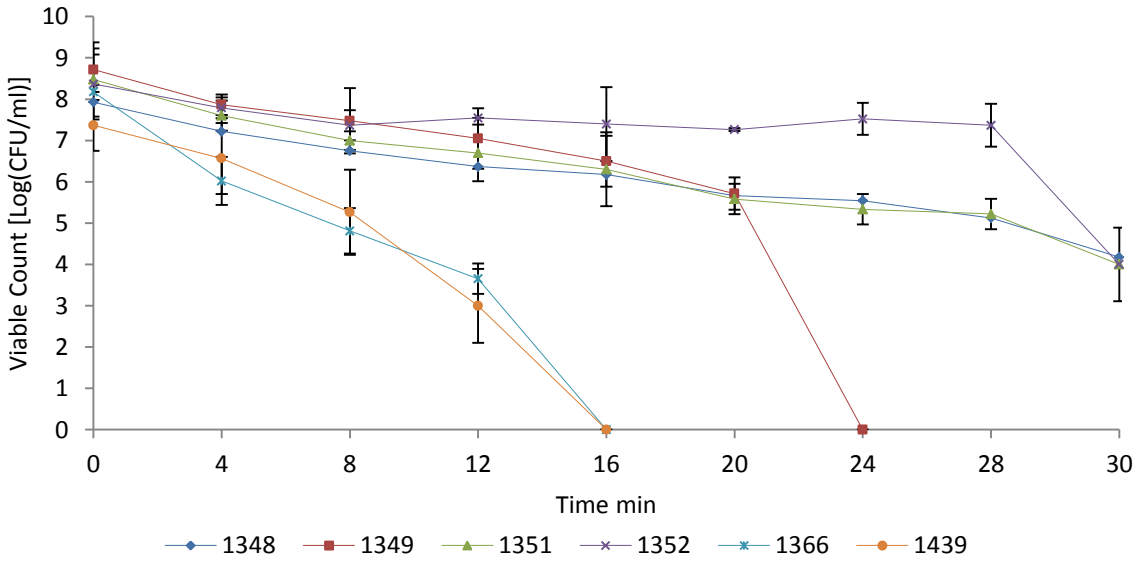
Ent. ludwigii

As shown in Table 4.1, *Ent.ludwigii* strain 1352 was more heat resistant than the remaining strains. It had a D-value of > 50 minutes at 55°C in whey and casein-based infant formula as presented in Figures 4.1 A and B. Whereas, *Ent.ludwigii* strain 1348 showed a very different death rate D_{55} =11.5 minutes in casein-based infant formula compared with D_{55} =5 minutes in whey-based infant formula. *Ent. ludwigii* strains 1349 and 1351 showed moderate heat resistance and the viability decline was linear in casein-based infant formula (D values were D_{55} = 6 and 9.5 minutes respectively). Strain 1349 showed clear difference in survival in whey-based infant formula with $D_{55} \leq 4$ minutes, and was deemed sensitive to heat. Furthermore, *Ent. ludwigii* strains 1366 and 1439 showed sensitivity to heat and subsequently had lower D-values in whey and casein-

based infant formula (Table 4.1, Figures 4.1 B and B). In general, *Ent. ludwigii* heat treated in whey-based infant formula product had similar D- values to those determined in the casein-based infant formula product. However, in casein-based infant formulas the *Ent.ludwigii* strains were more heat tolerant and had shorter log-periods.



(A)



(B)

Figure 4.1: Survival of *Ent. ludwigii* in (A) whey-based infant formul and (B) in casein-based infant formula at 55°C.

Ent. Ludwigii isolates survived for different length time in whey-based infant formula compered to casein-based infant formula. *Ent. Ludwigii* isolates 1348, 1349, 1351, 1352, 1366 and 1439 were incubated in either whey-based (Panel A) or casein-based (Panel B) formula for 30 minutes at 55°C. Aliquots were removed at the indicated times for cfu 1 log (D value) is indicated in Table 4.1. Experiments were performed in duplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation.

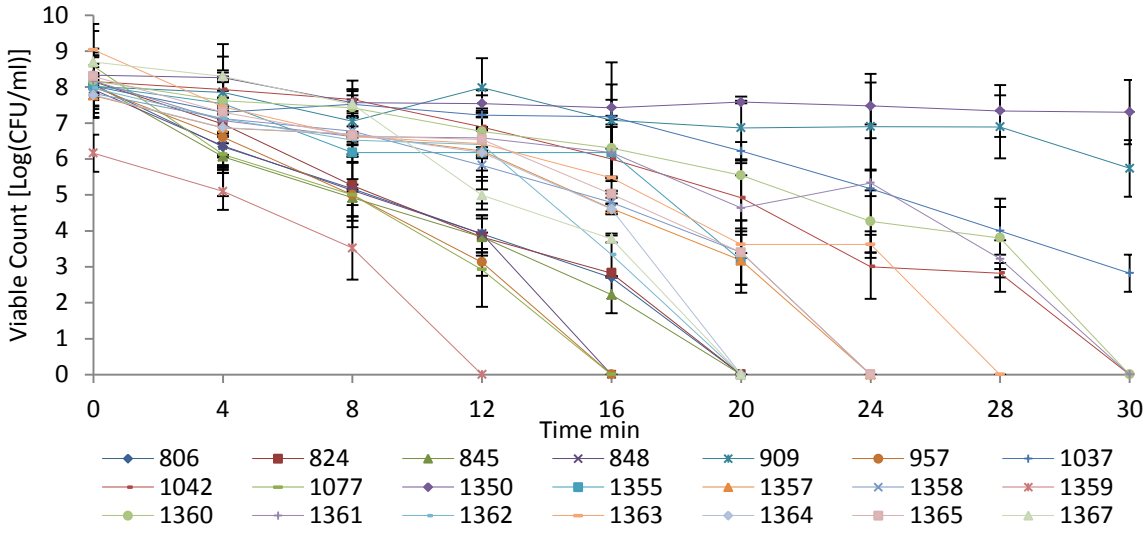
Ent. cancerogenus

There was variation in thermotolerance between *Ent. cancerogenus* strains. *Ent. cancerogenus* strains were differentiated into three groups based on their capability of heat resistance.

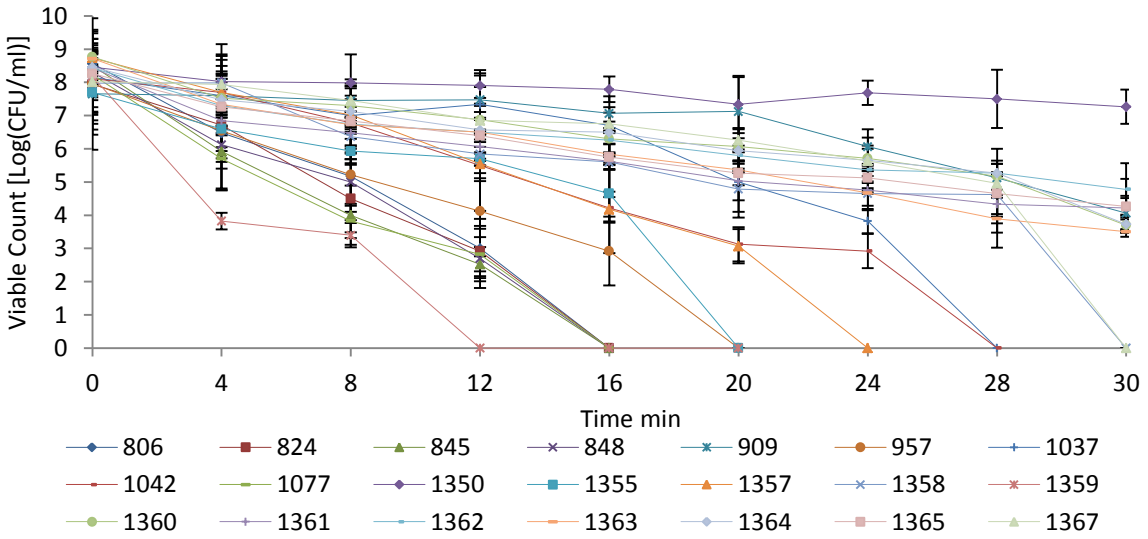
Heat resistant: *Ent. cancerogenus* strains 909 and 1350 showed high thermoresistance in whey-based infant formula and moderate heat resistance in casein-based infant formula with D_{55} >50 minutes, (Figures 4.2 A and B). In contrast, strain 1367 showed heat resistance at 55ºC in casein and had moderate heat resistance in whey.

Heat moderately tolerant: The *Ent. cancerogenus* strains 1037, 1355, 1358, 1360-1362 and 1365 were moderately resistant to heat at 55°C in whey and casein-based infant formula, as seen in Table 4.1.

Heat sensitive: *Ent. cancerogenus* strains 806, 824, 845, 848, 957, 1042, 1077, 1357, 1359, 1363 and 1364 were more sensitive to heat in both whey and casein-based infant formula except strain 1042 and 1357 which were moderate to heat in whey-based infant formula with D_{55}= 6.5 and 6 minutes, respectively. (See Figure 4.2 A and B, Table 4.1).



(A)



(B)

Figure 4.2: Survival of *Ent. cancerogenus* in (A) whey-based infant formul and (B) in casein based-infant formula at 55°C.

Ent. cancerogenus isolates survived for different length time in whey-based infant formula compered to casein-based infant formula. *Ent. cancerogenus* isolates 806, 824, 845, 848, 909, 957, 1037, 1042, 1077, 1350, 1355, 1357, 1358, 1359, 1360, 1361, 1362, 1363, 1364, 1365, and 1367 were incubated in either whey-based (Panel A) or casein-based (Panel B) formula for 30 minutes at 55°C. Aliquots were removed at the indicated times for cfu 1log (D value) is indicated in Table 4.1. Experiments were performed in duplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation

Ent. hormaechei

Ent. hormaechei strains 1075, 1053 and 1038 showed more heat tolerance (resistant) at 55°C in whey and casein-based infant formula. The D-value of *Ent. hormaechei* strains 1075 and 1038 were D_{55}= 11, 20 and >50 minutes in whey, and 11, 8.5 and >50 minutes in casein respectively. However, *Ent. hormaechei* strain 1053 showed high resistance in whey only, while demonstrating moderately tolerant resistance to heat in casein-based infant formula.

Ent. hormaechei strain 790 was moderately tolerant to heat in whey and casein based-infant formula, and the D-value was 6.5 and 5 minutes respectively.

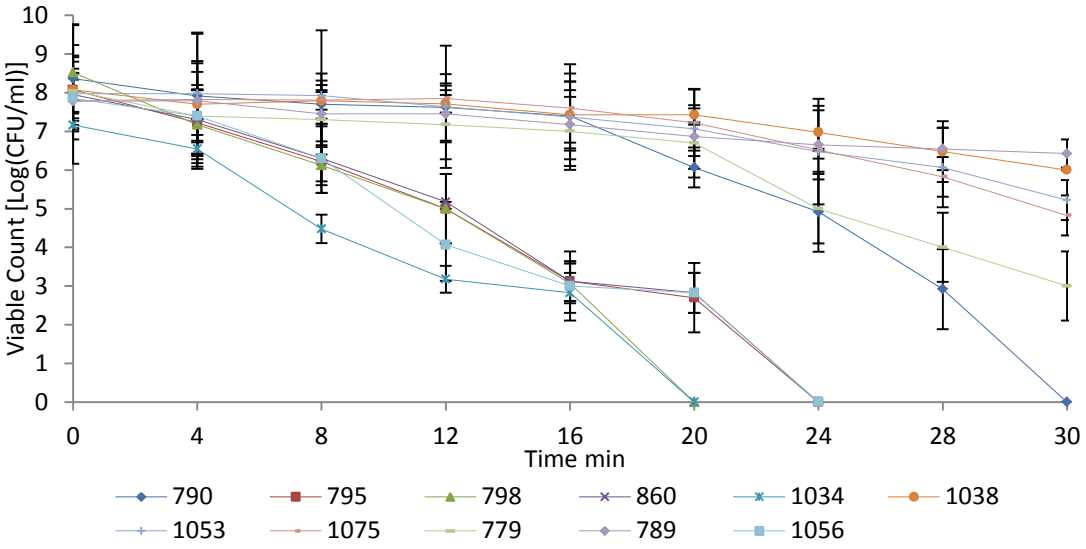
The sensitivity of *Ent. hormaechei* strains 795, 798, 860 and 1034 to heat results showed a high rate of death to 55°C in whey and casein-based infant formula. The D-values for these organisms at 55°C were less then 4 minutes.

Ent. cloacae

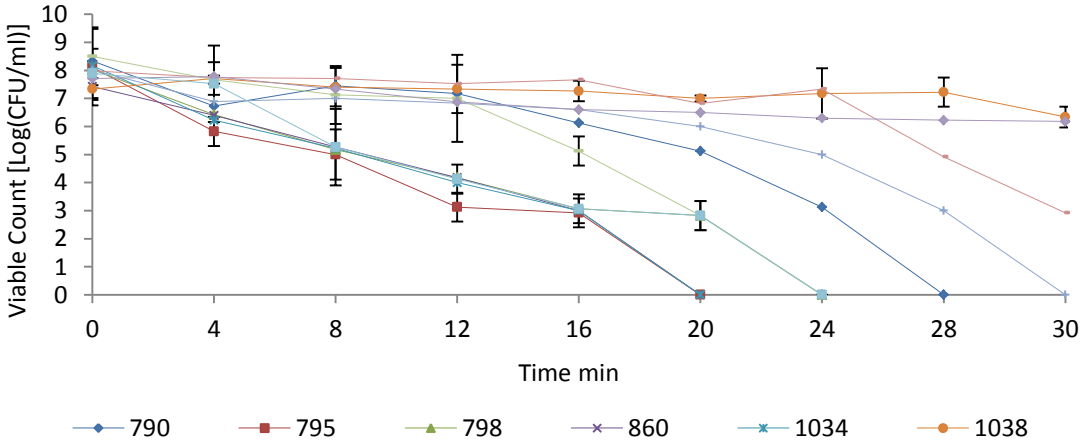
Two strains of *Ent. cloacae*, 779 and 789, showed variation between contradictory moderate heat tolerance and resistance at 55ºC in whey and casein-based infant formula; the exception was strain 779 which was moderately tolerant to heat in casein-based infant formula with D_{55} > 10 minutes; (see Figure 4.3 A and B, Table 4.2).

Ent. aerogenes

Ent. aerogenes strain 1056 was more sensitive to heat in both whey and casein-based infant formula. The D-values for this organism at 55°C were 2 minutes in both types of milk; (see Figure 4.3 A and B, Table 4.2).



(A)



(B)

Figure 4.3: Survival of *Ent. hormaechei*, *Ent. cloacae* strains (779 and 789) and *Ent. aerogenes* strain 1056 in (A) whey-based infant formul and (B) in casein-based infant formula at 55°C.

Ent. hormaechei, *Ent. cloacae* and *Ent. aerogenes* isolates survived for different length time in whey-based infant formula compered to casein-based infant formula. *Ent. hormaechei* isolates 790, 795, 798, 860, 1034, 1038, 1053 and 1075, *Ent. cloacae* isolates 779 and 789 and *Ent. aerogenes* isolate 1056 were incubated in either whey-based (Panel A) or casein-based (Panel B) formula for 30 minutes at 55°C. Aliquots t were removed at the indicated times for cfu 1 log (D value) is indicated in Table 4.2. Experiments were performed in duplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation.

K. oxytoca

Differences in the survival of *K. oxytoca* at 55ºC were noted in the two types of formula (whey and casein-based infant formula). The *K. oxytoca* strain 1356 was thermoresistant at 55ºC in whey and casein-based infant formula with a D-value more than 50 minutes and was nonlinear. Whereas moderate resistance to heat in whey based infant formula was demonstrated by *K. oxytoca* strain 1353 with D_{55} =5.5, and sensitive to heat in casein-based infant formula with a D-value undetected D_{55}=3 minutes; (presented in Figure 4.4 A and B, Table 4.2).

K. oxytoca strain 1354 showed moderate resistance to heat in whey and casein-based infant formula.

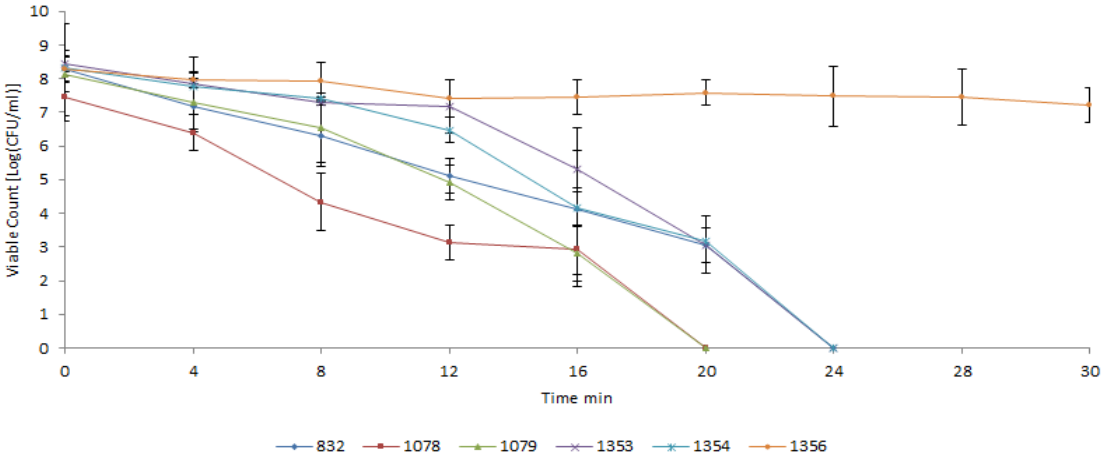
In Table 4.2, Figures 4.4 A and 4.4 B the *K. oxytoca* strains 832, 1078 and 1079 showed sensitive behavior to heat at 55ºC in whey and casein-based infant formula and the D-values were not detected before 4 minutes.

Summary

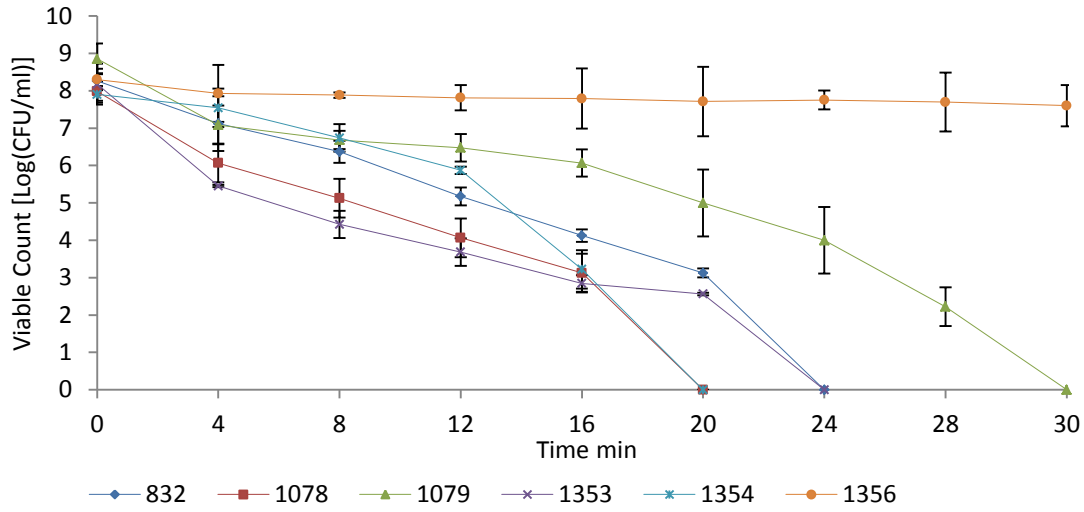
In this study across all the bacterial species,1/6 strain of *Ent. ludwigii*, 2/21 strains of *Ent. cancerogenus* and 1/8 strains of *Ent. hormaechei* and 1/6 strains of *K. oxytoca* showed the highest thermal resistance on both based infant formula at 55°`C. These strains were not affected by both formula types at 55°`C.

Clonally related strains (same pulsotype) showed the same death rate at 55ºC. For instance, *Ent. cancerogenus* strains 845, 848, 1363, 1364, 795 and 798 and *K. oxytoca* strains 1078 and 1079 were sensitive to heat tolerance at 55ºC on whey and casein-based infant formula. These belong to four clusters; Enc1, Enc3, Ebh6 and Kc3 with each pulsotype comprising two strains respectively (Tables 4.1 and 4.2).

Also, two strains (1355, 1358) of *Ent. cancerogenus* showed the same response to heat at 55ºC in whey and casein-based infant formula, which was moderately resistant, and they belonged to the same pulsotype (Enc2); see Table 4.1.



(A)



(B)

Figure 4.4: Survival of *K. oxytoca* in (A) whey-based infant formul and (B) in casein-based infant formula at 55°C.

K. oxytoca isolates survived for different length time in whey-based infant formula compered to casein-based infant formula. *K. oxytoca* isolates 832, 1078, 1079, 1353, 1354 and 1356 were incubated in either whey-based (Panel A) or casein-based (Panel B) formula for 30 minutes at 55°C. Aliquots were removed at the indicated times for cfu 1 log (D value) is indicated in Table 4.2. Experiments were performed in duplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation.

Table 4.1: D-value for *Ent. ludwigii* and *Ent. cancerogenus* strains in two different types of formula milk.

Species	Strain	Source of isolates	Neonatal No.	PFGE cluster	Formula	D_{55} (min)	Category
Ent.ludwigii	1348	MBM	Unknown	U	casein	11.5	M
					whey	5	M
"	1349	„	Unknown		casein	6	M
					whey	4	M
"	1351	„	Unknown		casein	9.5	M
					whey	5	M
"	1352	„	Unknown		casein	> 50	R
					whey	> 50	R
"	1366	„	Unknown		casein	2.5	S
					whey	1	S
"	1439	CSF	Unknown		casein	2	S
					whey	1	S
Ent.cancerogenus	806	EFT	24	U	casein	2	S
					whey	2	S
"	824	„	36	Ebc3	casein	1	S
					whey	2	S
"	845	„	45	Ebc1	casein	1	S
					whey	2	S
"	848	„	48		casein	2.5	S
					whey	2.5	S
"	909	„	58	Ebc2	casein	> 50	R
					whey	> 50	R
"	957	„	81	Ebc5	casein	1	S
					whey	1	S
"	1037	„	112	Ebc6	casein	8	M
					whey	9	M
"	1042	„	128	Ebc4	casein	3.5	S
					whey	6.5	M
"	1077	„	118	U	casein	1.5	S
					whey	2.5	S
"	1350	MBM	Unknown		casein	> 50	R
					whey	> 50	R
"	1357	„	Unknown		casein	3.5	S
					whey	6	M
"	1358	„	Unknown	Enc2	casein	7.5	M
					whey	6.5	M
"	1355	„	Unknown		casein	7.5	M
					whey	8	M
"	1359	„	Unknown	Enc1	casein	0.5	S
					whey	3.5	S
"	1360	„	Unknown		casein	8.5	M
					whey	6	M
"	1361	„	Unknown	U	casein	7	M
					whey	9	M
"	1362	„	Unknown		casein	10.5	M
					whey	4	M
"	1363	„	Unknown	Enc3	casein	3.5	S
					whey	4	M
"	1364	„	Unknown		casein	2	S
					whey	1	S
"	1365	„	Unknown	U	casein	9	M
					whey	6	M
"	1367	„	Unknown		casein	19	R
					whey	5	M

EFT: Indicates enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates cerebrospinal fluid. Ebc1-6 and Enc1-3indicates PFGE cluster groups, U: Unique. D- value indicate the time required for a reduction in the viable count at by 1 log. R, M and S indicate resistant, moderate and sensitive strains to heat tolerance at 55ºC respectively.

Table 4.2: D-value for *Ent. hormaechei* and *K. oxytoca* in two different types of formula milk**.**

Species	Strain	Source of isolates	Neonatal No.	PFGE cluster	Formula	D_{55} (min)	Category
Ent. hormaechei	790	EFT	4	U	casein	5	M
					whey	6.5	M
"	795	„	17	Ebh6	casein	2	S
					whey	2	S
"	798	„	18		casein	2	S
					whey	1	S
"	860	„	52	Ebh4	casein	1	S
					whey	1.5	S
"	1034	„	110	Ebh2	casein	2.5	S
					whey	1.5	S
"	1038	„	113	Ebh7	casein	> 50	R
					whey	> 50	R
"	1053	„	117	Ebh3	casein	8.5	M
					whey	20	R
"	1075	„	117		casein	11	M
					whey	11	M
Ent. cloacae	779	EFT	2	Ebl1	casein	4	M
					whey	> 50	R
"	789	„	12		casein	13	M
					whey	10.5	M
Ent. aerogenes	1056	EFT	118	Eba1	casein	2	S
					whey	2	S
K.oxytoca	832	EFT	36	Kc5	casein	3	S
					whey	2	S
"	1078	„	120	Kc3	casein	2.5	S
					whey	2.5	S
"	1079	„	121		casein	2	S
					whey	1	S
"	1353	MBM	Unknown	U	casein	3	S
					whey	5.5	M
"	1354	„	Unknown		casein	6.5	M
					whey	4.5	M
"	1356	„	Unknown		casein	> 50	R
					whey	> 50	R

EFT: Indicates enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates cerebrospinal fluid. Ebh2-4, 6 and 7; Ebl1; Eba1 and Kc3 and 5 indicates PFGE cluster groups, U: Unique. D- value indicate the time required for a reduction in the viable count at by 1 log. R, M and S indicate resistant, moderate and sensitive strains to heat tolerance at 55ºC respectively.

4.3.2 Biofilm formation by *Enterobacteriaceae* on three types of formula milk at different temperatures

The biofilm formation values had to be corrected for the non-specific binding of crystal violet to the microtitre tray and un-inoculated formula. The relative biofilm formation value was determined by dividing the strain value by the corresponding control value. The results were then categorised as:

1- No biofilm formation: OD strain = OD control
2- Low biofilm formation: OD strain ≤ 2x OD control
3- Moderate biofilm formation: OD strain ≤ 4x OD control
4- High biofilm formation: OD strain > 4x OD control.

Twenty strains isolated from MBM and twenty three strains isolated from EFT (Tables 2.2 and 2.4 respectively) in section 2.1.1 were tested for biofilm formation in three types of formula milk (whey, casein and soya-based infant formula) at 20°C and 37°C.
The results are summarised below and summarised in Table 4.3.

4.3.2.1 Whey-based formula

- ***At 20°C:***

Twenty four out of fourty four strains were able to form biofilm at 20°C including 4/6 strains of *Ent. ludwigii,* 4/8 strains of *Ent. hormaechei,* 2 strains of *Ent. cloacae,* 17/21 strains of *Ent. cancerogenus* and 3/6 strains *K. oxytoca* formed relatively low levels of biofilm. The rest of strains were not able to form biofilm at 20°C; (Figure 4.9).

- ***At 37°C:***

All strains formed biofilms on whey-based infant formula at 37°C. The high biofilm level was produced by *Ent. ludwigii* strain 1439 and *K. oxytoca* strain 1353. Moderate levels of biofilm were produced by 3/6 strains of *Ent. ludwigii*, 6/8 strains of *Ent. hormaechei*, 2 strains of *Ent. cloacae*, 1 strain of *Ent. aerogenes*, 17/21 strains of *Ent. cancerogenus* and 4/6 strains of *K. oxytoca*. The remaining strains only formed low levels of biofilm; (see Figure 4.9).

Interestingly, the same level of biofilm was observed by *Ent. hormaechei* strains 1053 and 1075 in whey at 37ºC which were isolated from the same patient and belonged to pulsotype Ebh3; (Table 4.3). In addition, *Ent. cancerogenus* strains (1355 and 1358) and *Ent. cancerogenus* strains (1359 and 1360) showed moderate biofilm level in whey-based infant formula at 37°C and belonged to pulsotypes Enc2 and Enc1 respectively. *Ent. cancerogenus* 1363 and 1364 also showed moderate biofilm formation and belonged to pulsotype Enc3 (Table 4.3).

The biofilm formation at 37°C was significantly higher than the formation at 20°C ($p<0.05$).

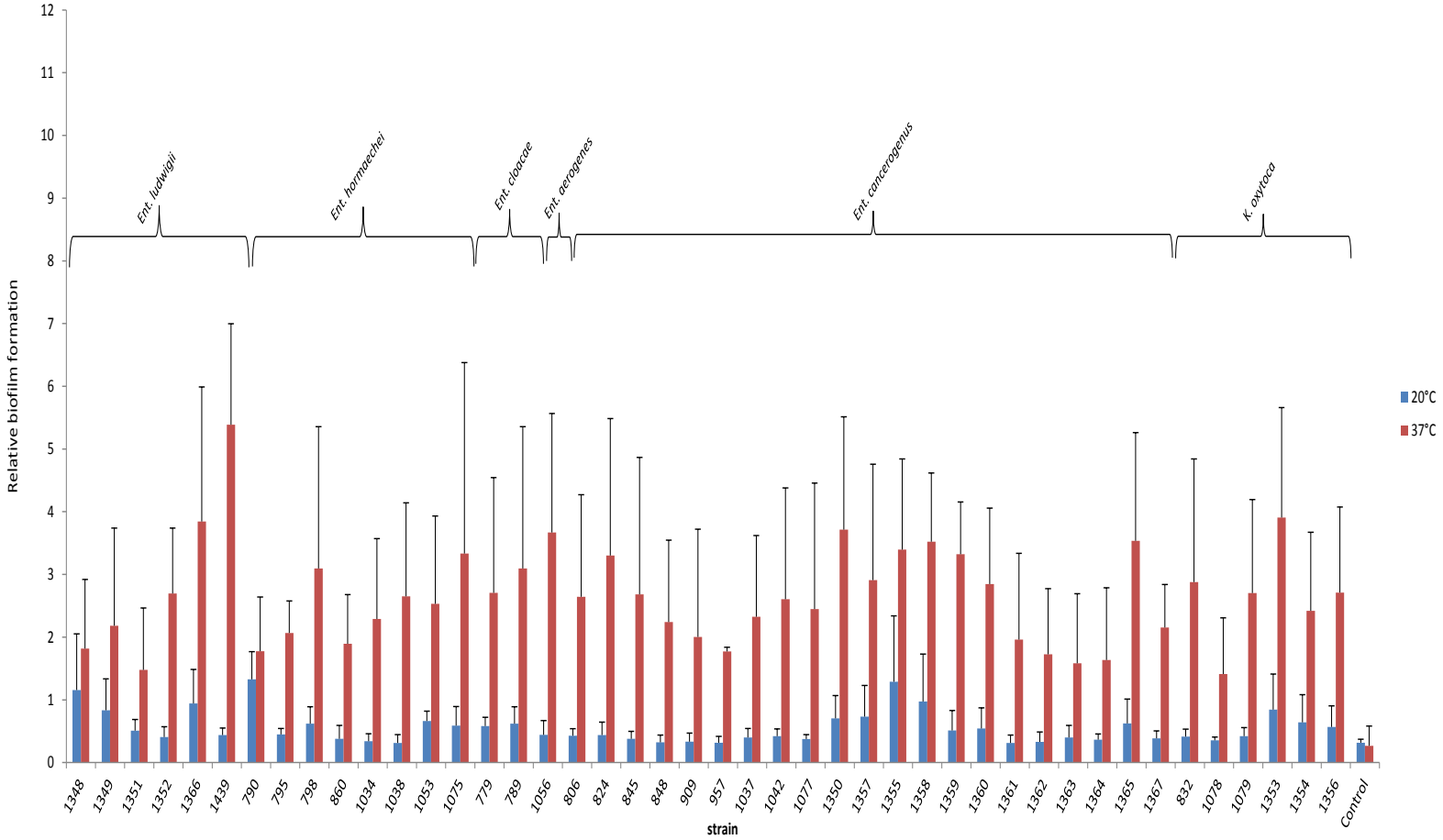


Figure 4.5: : Biofilm formation in whey-based infant formula at 20°C and 37°C.

Experiments were performed in triplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation of two independently repeated experiment.

4.3.2.2 Casein-based formula

- ***At 20°C:***

There was no high biofilm on casein-based infant formula at 20°C. Moderate biofilms were produced by 1/6 strains of *Ent. ludwigii,* 2/8 strains of *Ent. hormaechei* and 3/21 strains of *Ent. cancerogenus*. Low level of biofilm was formed by 5/6 strains of *Ent. ludwigii,* 1/8 strains of *Ent. hormaechei*, two strains of *Ent. cloacae*, 3/21 strains of *Ent. cancerogenus* and 1/6 strains of *K. oxytoca*. The rest of strains showed low biofilm at this temperature; see Figure 4.10.

- ***At 37°C:***

High biofilm formation was seen by 2/6 strains of *Ent. ludwigii,* 1/8 strains of *Ent. hormaechei,* 8/21 strains of *Ent. cancerogenus* and 1/6 strains of *K. oxytoca*. Moderate biofilm levels were formed by 1/6 strains of *Ent. ludwigii*,, 3/8 strains of *Ent. hormaechei*, 2 strains of *Ent. cloacae,* 1 strain of *Ent. aerogenes*, 8/21 strains of *Ent. cancerogenus* and 3/6 strains of *K. oxytoca* (Figure 4.10, Table 4.3).

Low biofilm levels were produced by 3/6 strains of *Ent. ludwigii,* 4/8 strains of *Ent. hormaechei*, 5/21 strains of *Ent. cancerogenus* and 2 /6 strains of *K. oxytoca*; (Figure 4.10). The difference in the quantity of biofilm formation between the two temperatures (20°C and 37°C) was statistically significant in most strains. Results are summarised in Figure 4.10 and Table 4.3.

It was also noted that most strains which belonged to the same pulsotype showed the same biofilm result; for example, *Ent. cancerogenus* strains 1359 and 1360 belonged to cluster Enc1 and formed high biofilm on infant formula at 37°C and produced low biofilm at 20°C. Also, *Ent. cancerogenus* strains 1363 and 1364 belonged to group Enc3 which formed low biofilm at 20°C and 37°C (Table 4.3).

4.3.2.3 Soya-Powder Infant formula

- ***At 20°C:***

None of the tested strains produced high levels of biofilm formation in soya milk at both temperatures 20°C and 37°C. There was low biofilm formation by all *Ent. ludwigii,*

Ent. hormaechei, *Ent. cloacae*, *Ent. aerogenes*, *Ent. cancerogenus* and *K. oxytoca* strains at 20°C (Figure 4.11, Table 4.3).

- ***At 37°C:***

Three *Ent. cancerogenus* strains (1355, 1357 and 1358) and 1 *K. oxytoca* strain (1356) formed moderate biofilm levels at 37°C, and low biofilm was formed by all strains of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cloacae*, *Ent. aerogenes*, *Ent. cancerogenus* and *K. oxytoca* strains at 37°C (see Figure 4.11, Table 4.3).

Biofilm formation in soya milk was statistically significantly different at 20°C and 37°C ($p<0.05$).

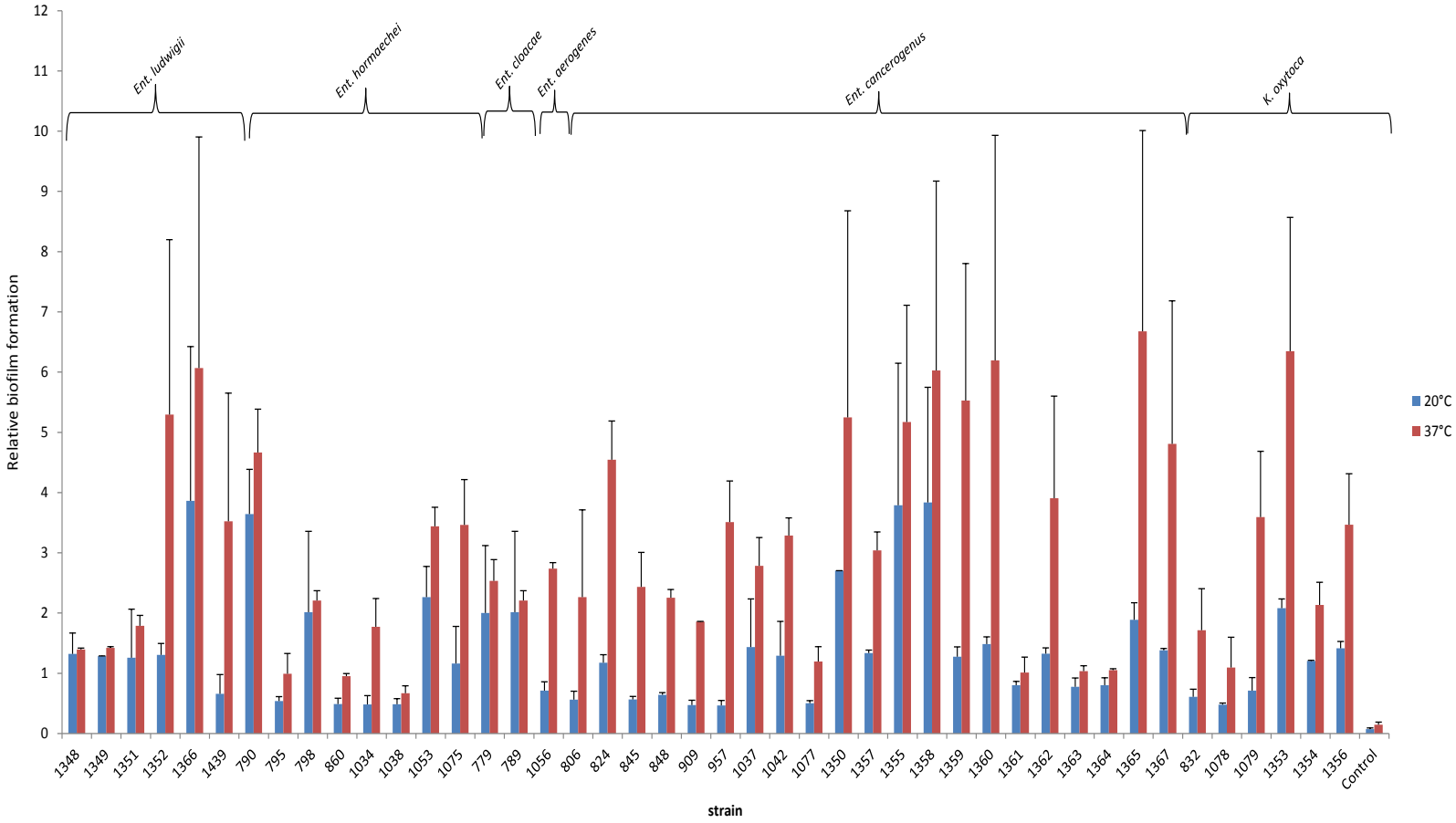


Figure 4.6: Biofilm formation in casein-based infant formula at 20°C and 37°C.

Experiments were performed in triplicate and data are presented as the average of two independent experiments. Error bars represent standard deviation of two independently repeated experiment.

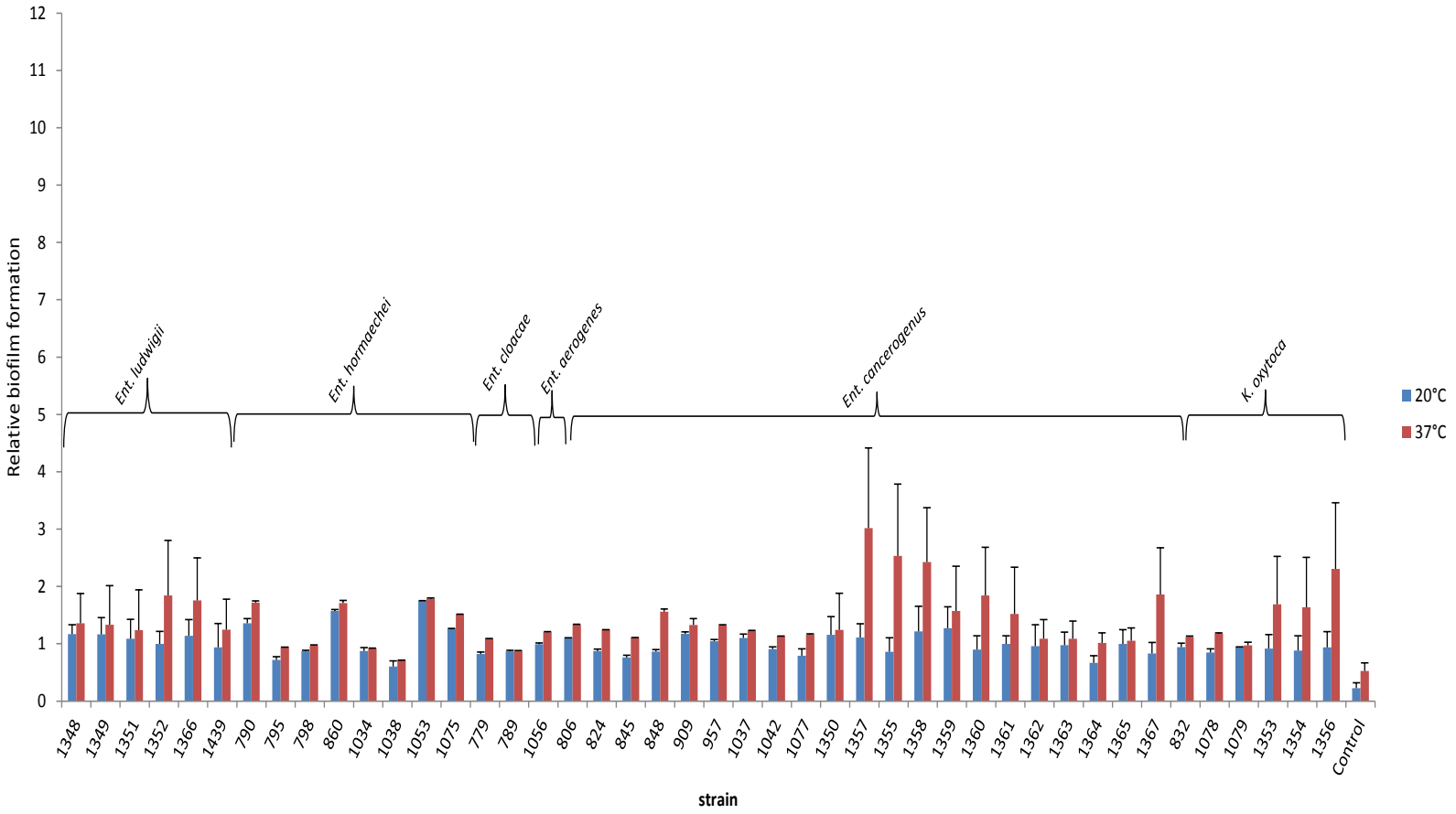


Figure 4.7: Biofilm formation in soya powder infant formula at 20°C and 37°C.

Experiments were performed in triplicate and data are presented as the average of two independent experiments. Error bars represents standard deviation of two independently repeated experiment.

4.3.2.4 Comparison of different levels of biofilm formation at 37°C

In *Ent. ludwgii*, biofilm formation was significantly higher in casein and whey-based infant formula than soya-based infant formula ($p < 0.001$), while there was no significant difference between casein and soya-based formula.

In *Ent. cancergenus*, biofilm formation was significantly higher in casein based-infant formula than both whey and soya-based infant formula ($p < 0.001$); however, the biofilm level was significantly higher in whey compared with soya-based formula.

In *Ent. hormaechei* and *K. oxytoca*, biofilm formation was significantly higher in casein and whey-based infant formula than soya-based infant formula ($p<0.001$).

In *Ent. cloacae* and *Ent. aerogenes*, biofilm formation was not significantly different between the three types of formula (whey, casein and soya) .

In summary, it was found that the relative biofilm formation was higher at 37ºC compared with 20ºC by most tested strains in all types of formula (Table 4.3). The quantity of biofilm was detected on three types of formula which was higher on casein-based infant formula compared with whey and soya-based infant formula in all strains.

Table 4.3: Biofilm formation level on based-infant formula (whey, casein and soya) by EFT strains and MBM strains.

Species	Strain	Source of isolates	Neonatal No.	PFGE cluster	Biofilm formation					
					whey		casein		soya	
					20°C	37°C	20°C	37°C	20°C	37°C
Ent. ludwigii	1348	MBM	Unknown	U	L	L	L	L	L	L
„	1349	„	Unknown		L	M	L	L	L	L
„	1351	„	Unknown		N	L	L	L	L	L
„	1352	„	Unknown		N	M	L	H	L	L
„	1366	„	Unknown		L	M	M	H	L	L
„	1439	CSF	Unknown		L	H	L	M	L	L
Ent. hormaechei	790	EFT	4	U	L	L	M	H	L	L
„	795	„	17	Ebh6	N	M	L	L	L	L
„	798	„	18		L	M	L	M	L	L
„	860	„	52	Ebh4	N	L	L	L	L	L
„	1034	„	110	Ebh2	N	M	L	L	L	L
„	1038	„	113	Ebh7	N	M	L	L	L	L
„	1053	„	117	Ebh3	L	M	M	M	L	L
„	1075	„	117		L	M	L	M	L	L
Ent. cloacae	779	EFT	2	Ebl1	L	M	M	M	L	L
„	789	„	12		L	M	M	M	L	L
Ent . aerogenes	1056	EFT	118	Eba1	N	M	L	M	L	L
Ent. cancerogenus	806	ETF	24	U	N	M	L	M	L	L
„	824	„	36	Ebc3	N	M	L	H	L	L
„	845	„	45	Ebc1	N	M	L	M	L	L
„	848	„	48		N	M	L	M	L	L
„	909	„	58	Ebc2	N	M	L	L	L	L
„	957	„	81	Ebc5	N	L	L	M	L	L
„	1037	„	112	Ebc6	N	M	L	M	L	L
„	1042	„	128	Ebc4	N	M	L	M	L	L
„	1077	„	118	U	N	M	L	L	L	L
„	1350	MBM	Unknown		L	M	M	H	L	L
„	1357	„	Unknown	U	L	M	L	M	L	M
„	1355	„	Unknown	Enc2	L	M	M	H	L	M
„	1358	„	Unknown		L	M	M	H	L	M
„	1359	„	Unknown	Enc1	L	M	L	H	L	L
„	1360	„	Unknown		L	M	L	H	L	L
„	1361	„	Unknown	U	N	M	L	N	L	L
„	1362	„	Unknown		N	L	L	M	L	L
„	1363	„	Unknown	Enc3	N	L	L	L	L	L
„	1364	„	Unknown		N	L	L	L	L	L
„	1365	„	Unknown	U	L	M	L	H	L	L
„	1367	„	Unknown		N	M	L	H	L	L
K. oxytoca	832	EFT	36	Kc5	N	M	L	L	L	L
„	1078	„	120	Kc3	N	L	L	L	L	L
„	1079	„	121		N	M	L	M	L	L
„	1353	MBM	Unknown	U	L	H	M	H	L	L
„	1354	„	Unknown		L	M	L	L	L	L
„	1356	„	Unknown		L	M	L	M	L	M

Summarised the biofilm formation level on three types of based infant formula (whey, casein and soya) by enteral feeding tube strains (EFT) and mastic human breast milk strains (MBM). CSF: indicates cerebrospinal fluid. Ebh2-4, 6 and 7; Ebl1 and Kc3 and 5 indicates PFGE cluster groups, U: Unique. Strains were categorised as follows: (N) No biofilm formation: OD strain = OD control, (L) low biofilm formation: OD strain ≤ 2x OD control, (M) Moderate biofilm formation: OD strain ≤ 4x OD control and (H) high biofilm formation: OD strain >4x OD control. EFT: Indicates Enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates Cerebrospinal fluid.

4.3.3 Capsule formation on milk agar

Bacterial capsule production was determined by colony appearance on milk agar (whey, casein and soya-based) at 20°C (after 24 hours and 48 hours) and 37°C (after 24 hours). The results were classified into three categories according to the relative scale of high, medium and low capsule production which are represented by ≥ +++, ++ and + respectively, Figure 4.8.

The amount of capsular material varied according to the incubation temperature, time, and milk agar composition; (Table 4.4). No highly mucoid colonies were apparent after 24 hours incubation at 20°C, but these did appear after 48 hours incubation. Capsular strains were found in *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cancerogenus*, and *K. oxytoca*. However, the number of capsular strains varied between species.

- On whey milk agar the capsule production was recorded for all *Ent. ludwigii* at 20ºC and 37ºC, except strain 1366 which produced medium capsular material at 37ºC only. The high level of capsular material was by 5/6 *Ent. ludwigii* at 37ºC and by 1 strain at 20ºC, whereas 4/6 *Ent. ludwigii* showed medium level of capsular material at 20ºC; see Table 4.4.

  With respect to *Ent. hormaechei*, 4/8 strains produced high capsular material on whey at 20ºC and 1 strain of *Ent. hormaechei* strain (1075) produced high capsule production at 37ºC. Three strains of *Ent. hormaechei* produced medium capsular material at 20ºC and 1 strain produced low capsular material at 37ºC (Table 4.4, Figure 4.8).

  Two strains of *Ent. cloacae* (779 and 789) formed low capsular material at 20ºC, while at 37ºC they produced medium/high capsular material respectively.

  Ent. aerogenes strain 1056 produced high capsular material at 20°C, but it was not able to form capsular material at 37°C; Table 4.4, Figure 4.8.

  Ent. cancerogenus strains 1364, 806, 957 and 1361 produced high capsular material at 20ºC, while at 37ºC the high capsular material was by strains 1364, 1361, 1357, 1363 and 1037. The majority of *Ent. cancerogenus*, 5/21 and 9/21 strains, produced medium capsular material at 20°C and 37°C respectively (Table 4.4, Figure 4.8). Of particular note was that most *Ent.*

cancerogenus produced capsular material at 37ºC.

Some *K. oxytoca* (2/6) strains produced high capsular material in whey-based infant formula at 37ºC and another 2/6 strains produced medium capsular material at 37ºC , and low capsular material was by *K. oxytoca* 3/6 and 1/6 strains at 20°C and 37°C respectively (Table 4.4, Figure 4.8). No capsule production was formed by 1/6 *Ent. ludwigii*, 11/21 *Ent. cancerogenus* and 1/6 *K. oxytoca* in whey-based infant formula at 20°C, while 6/8 *Ent. hormaechei*, 3/21 strains of *Ent. cancerogenus*, 1/6 *K. oxytoca* and 1 strain of *Ent. aerogenes* were not able to produce capsules in whey milk agar at 37°C. In contrast, *Ent. hormaechei* strain 798, *Ent. cancerogenus* strains 1358 and 909 and *K. oxytoca* strain 832 were not able to produce capsules in whey milk agar at both temperatures.

- On casein milk agar most species were able to produce capsular material at 20ºC and 37ºC. 3/6 and 5/6 *Ent. ludwigii* strains produced high capsular material at 20ºC and 37ºC respectively, while 3/6 and 1/6 *Ent. ludwigii* produced medium capsular material at 20ºC and 37ºC respectively. *Ent. hormaechei* strains 1034, 790, 860,1053 and 798 produced high capsular material at 20ºC, while strain 1075 formed high capsular material at 37ºC. Medium capsular material was formed by *Ent. hormaechei* strains 1075 and 860 at 20ºC and 37ºC respectively. *Ent. hormaechei* strain 1038 produced low capsular material at 37ºC. Of particular note was strain 1034 which produced considerable capsular material at 20ºC after 48 hours (Table 4.4, Figure 4.8).

  Ent. cloacae strains 779 and 789 formed low capsular material at 20ºC, whereas at 37ºC medium/high capsular material was produced respectively.
  The high capsular material was produced by *Ent. aerogenes* strain 1056 at 20°C; however, it was not able to produce capsular material at 37°C; (Table 4.4, Figure 4.8).

  The high capsular material was formed by 5/21 and 10/21 strains of *Ent. cancerogenus* at 20°C and 37ºC respectively. While the medium capsular material was produced by 8/21 and 10/21 strains of *Ent. cancerogenus* at 20°C and 37ºC respectively (Table 4.4, Figure 4.8).

K. oxytoca strains 1078, 1354 and 1356 produced high capsular material at 20°C and 37ºC. The exception was strain 1356 which formed low capsule at 20°C. The rest of strains produced medium capsular material at 37°C (Table 4.4, Figure 4.8).

- On soya powder infant formula agar the capsule production was produced by most species, the exception was by *Ent. cancerogenus* strain 909 which was not able to produce capsular material at both temperatures. 3/6 and 5/6 of *Ent. ludwigii* produced high capsular material at 20ºC and 37ºC respectively. All *Ent. hormaechei* produced high capsular material at 20ºC, while 5/8 of *Ent. hormaechei* formed high capsular material at 37ºC (Table 4.4, Figure 4.8).

  Ent. cloacae strains 779 and 789 produced medium capsular material at 20ºC, whereas at 37ºC these strains formed high/medium capsular material respectively. The high/medium capsular material was produced by *Ent. aerogenes* strain 1056 at 20°C and 37°C respectively (Table 4.4, Figure 4.8).

  The high capsule production was by 4/21 and 6/21 of *Ent. cancerogenus* at 20ºC and 37ºC respectively. Furthermore, 2/6 and 2/6 of *K. oxytoca* formed high capsular material at 20ºC and 37ºC respectively (Table 4.4, Figure 4.8).

  The ability of strains to produce capsular material at 20ºC and 37ºC was observed in casein-based infant formula and soya powder more than in whey-based infant formula and the capsular material production appeared on milk agar (whey, casein and soya based) at 20°C after 48 hours by most strains.
  Ent. ludwigii, *Ent. cancerogenus Ent. cloacae, Ent. aerogenes,* and *K. oxytoca* strains showed the ability to produce more capsular material on milk agar (whey, casein and soya based) at 20ºC and 37ºC, compared with *Ent. hormaechei* strain (see Table 4.4, Figure 4.8).

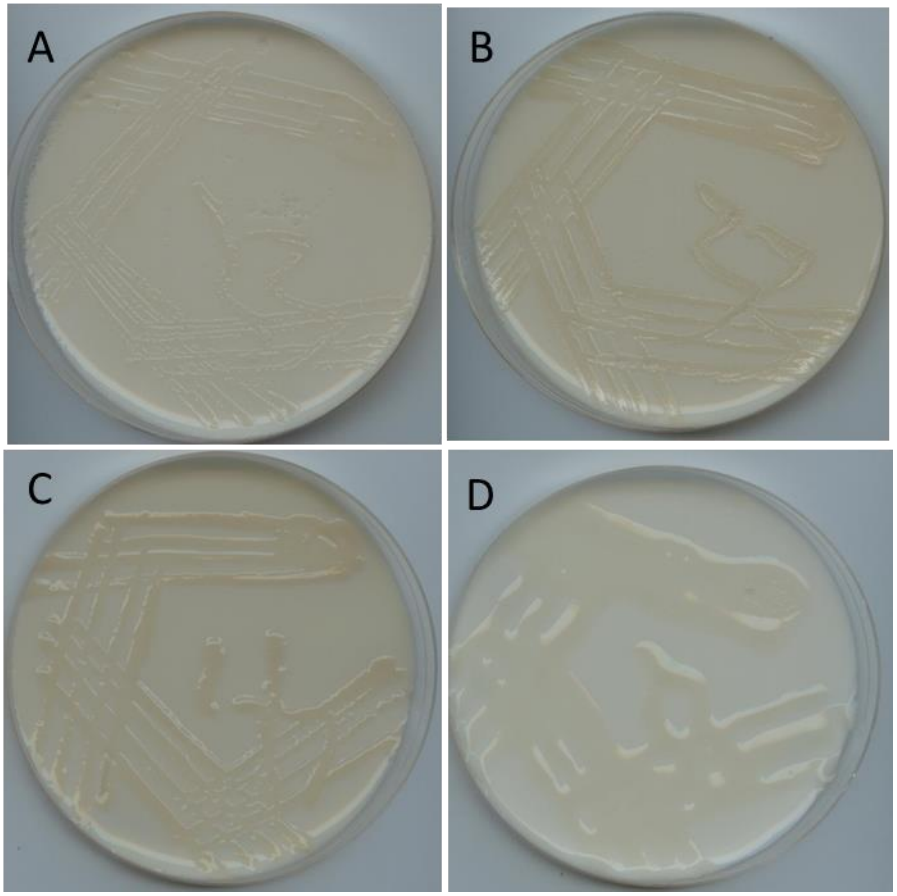


Figure 4.8: Capsule formation on milk agar by *Enterobacteriaceae* strains.

Capsular material classified into three categories according to the relative scale of high, medium and low capsule production. (A) indicates no capsule production; (B) indicates low capsule production; (C) indicates medium capsule and (D) indicates high capsule production. Capsular material was confirmed by India ink which appeared as a clear halo around the bacterium.

Table 4.4: Capsule production on based infant formula (whey, casein and soya) by EFT strains and MBM strains.

Species	Strain	Source of isolates	Capsule formation								
			whey			casein			soya		
			20°C 24h	20°C 48h	37°C 24h	20°C 24h	20°C 48h	37°C 24h	20°C 24h	20°C 48h	37°C 24h
Ent.ludwigii	1351	MBM	+	+++	++++	+	+++	++++	-	+++	+++
„	1439	CSF	-	++	+++	-	++++	+++	-	+++	+++
„	1352	MBM	-	++	+++	-	++	+++	-	+++	+++
„	1348	„	+	++	+++	+	++	+++	-	++	+++
„	1349	„	-	++	+++	-	++	+++	-	++	+++
„	1366	„	-	-	++	-	+++	++	-	++	++
Ent. hormaechei	1034	EFT	++	+++	-	+++	+++++	-	-	+++	+++
„	790	„	-	++++	-	-	++++	-	-	+++	++
„	860	„	++	+++	-	++	++++	++	-	+++	+++
„	1075	„	-	++	++++	-	++	+++	-	+++	++
„	1053	„	++	+++	-	++	+++	-	-	+++	+++
„	798	„	-	-	-	++	+++	-	-	+++	++
„	795	„	+	++	-	-	-	-	+	+++	+++
„	1038	„	++	++	+	-	-	+	-	+++	+++
Ent. cloacae	779	EFT	+	+	++	+	+	+++	-	++	+++
„	789	„	+	+	+++	+	+	++	-	++	++
Ent . aerogenes	1056	EFT	++	+++	-	+	+++	-	-	+++	++
Ent. cancerogenus	1364	MBM	+	+++	++++	-	+++	++++	-	+	++
„	806	EFT	++	++++	++	-	++	++++	+	++	+++
„	1361	MBM	-	++++	++++	-	++++	+++	+	+++	++
„	1357	„	+	++	+++	++	++	+++	-	++	+++
„	1363	„	-	-	++++	-	++	++++	+	++	++
„	957	EFT	++	+++	++	+	+++	++	-	+++	++
„	1360	MBM	+	++	++	+++	++++	++	-	++	++
„	1350	„	-	-	++	-	++	+++	+	+++	+++
„	1365	„	-	-	++	+	++	++	-	+	++
„	1359	„	-	++	++	-	++	+++	-	+	+++
„	1077	EFT	-	++	+	-	+++	+++	-	+	-
„	1037	„	-	-	+++	-	-	+++	+	+++	+++
„	824	„	-	-	++	-	-	++	-	++	++
„	845	„	-	-	+	-	-	++	-	++	++
„	848	„	-	-	++	-	-	++	-	++	+
„	1362	MBM	-	+	++	-	+	++	-	++	++
„	1042	EFT	+	++	-	+	++	++	-	++	+++
„	1355	MBM	-	-	+	-	++	++	-	+	++
„	1367	„	-	-	+	-	+	++	-	+	++
„	1358	„	-	-	-	-	+	+++	-	+	++
„	909	„	-	-	-	-	-	-	-	-	-
K. oxytoca	1078	EFT	-	+++	+	-	+++	++++	-	+	++
„	1354	MBM	-	+	++++	+	+++	+++	-	+++	+++
„	1356	„	+	+	++	+	+	++++	-	+++	++
„	1079	EFT	-	-	+++	-	-	++	-	+	++
„	1353	MBM	-	+	++	-	+	++	-	++	++
„	832	EFT	-	-	-	-	-	++	-	++	+++

Summarised the results of capsular material divided into three categories according to the relative scale of high, medium and low capsule production which are represented by ≥ +++, ++ and + respectively.(-) indicates no capsule production. EFT: indicates enteral feeding tube, MBM: indicates human mastic breast milk and CSF: indicates Cerebrospinal fluid.

4.3.3 Acid tolerance of organisms to pH 3.5 (HCl acidified formula)

The ability of *Ent. ludwigii*, *Ent. hormaechei*, *cloacae*, *Ent. aerogenes*, *Ent. cancerogenus* and *K. oxytoca* to tolerate acidic conditions at pH 3.5 was determined. These strains were exposed to two types of formula (whey and casein) which were adjusted to pH 3.5 for two hours at body temperature.

All strains were clearly resistant to two hours exposure to pH 3.5 in whey and casein infant formula: the viable counts were observed over the course of the 2 hours exposure to pH 3.5 for all strains. The variation across the strains in each type of formula was very broad. There was no correlation between the source of the strains (that is, enteral feeding tube isolated compared with mastic human breast milk strains) and their acid resistance. The results are presented in Figures 4.12-4.19. These results will serve as a base for bacterial attachment and invasion of host cells studies in chapter 5. The ability of organisms to survive at pH 3.5 (similar to the acidic environment of the stomach) means that these organisms are most likely to be pathogenic and able to invade the intestine, thus causing human infections.

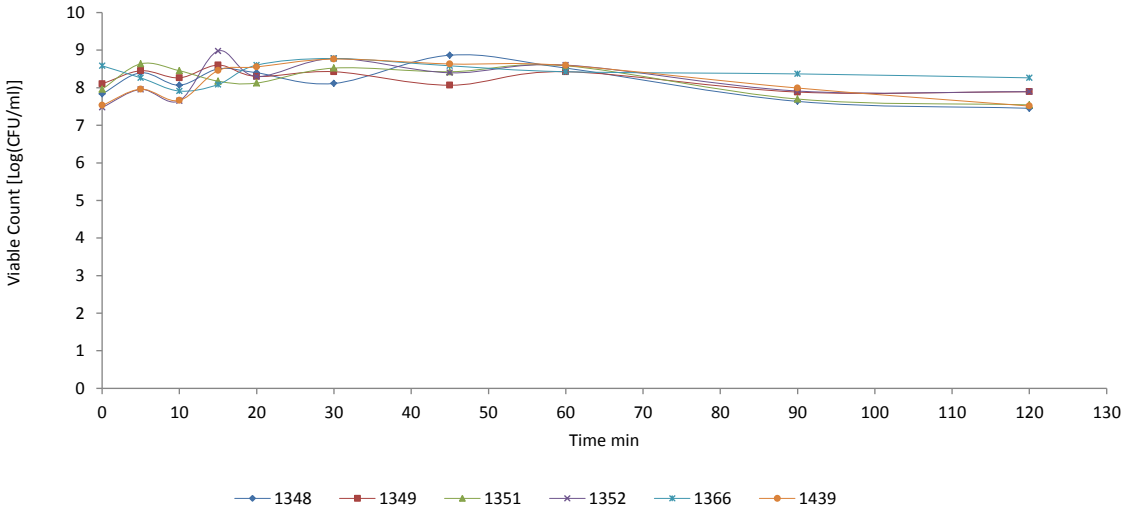


Figure 4.9: Survival of *Ent. ludwigii* in whey-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

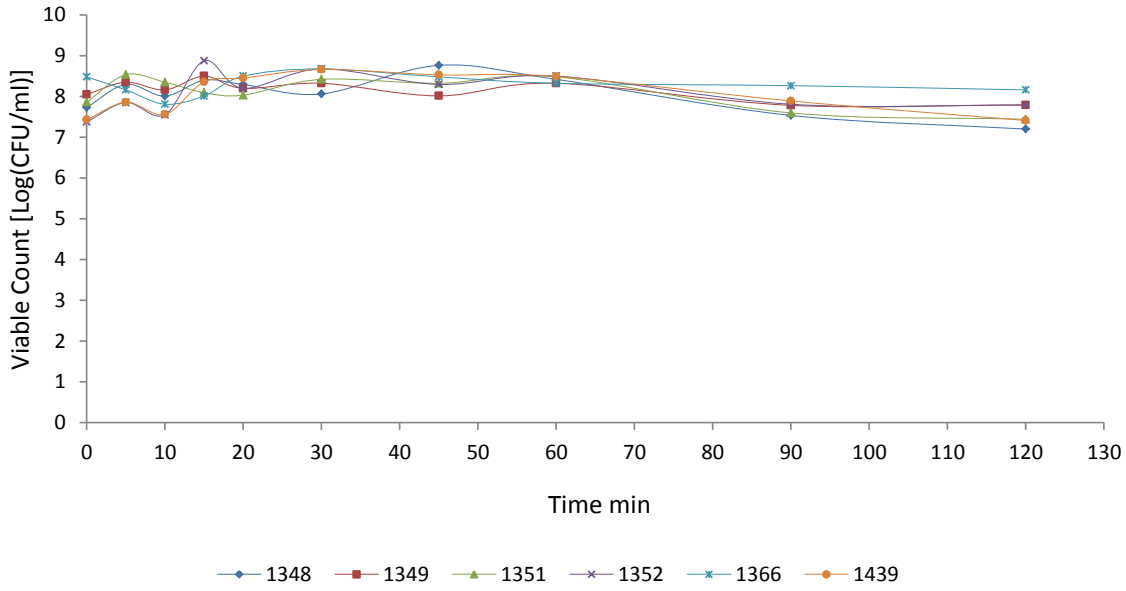


Figure 4.10: Survival *Ent. ludwigii* in casein-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

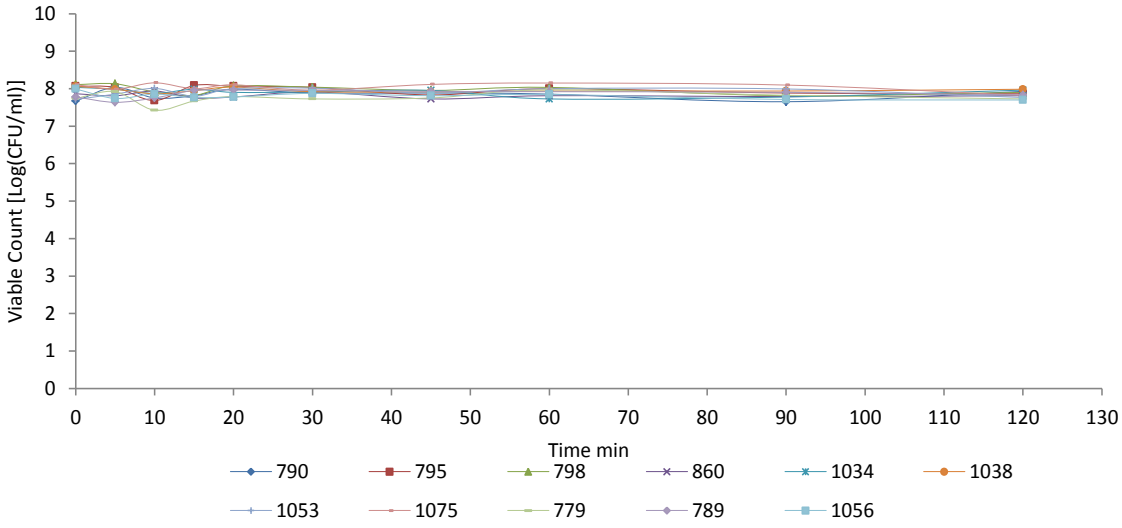


Figure 4.11: Survival of *Ent. hormaechei*, *Ent. cloacae* and *Ent.aerogenes* strains in whey-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

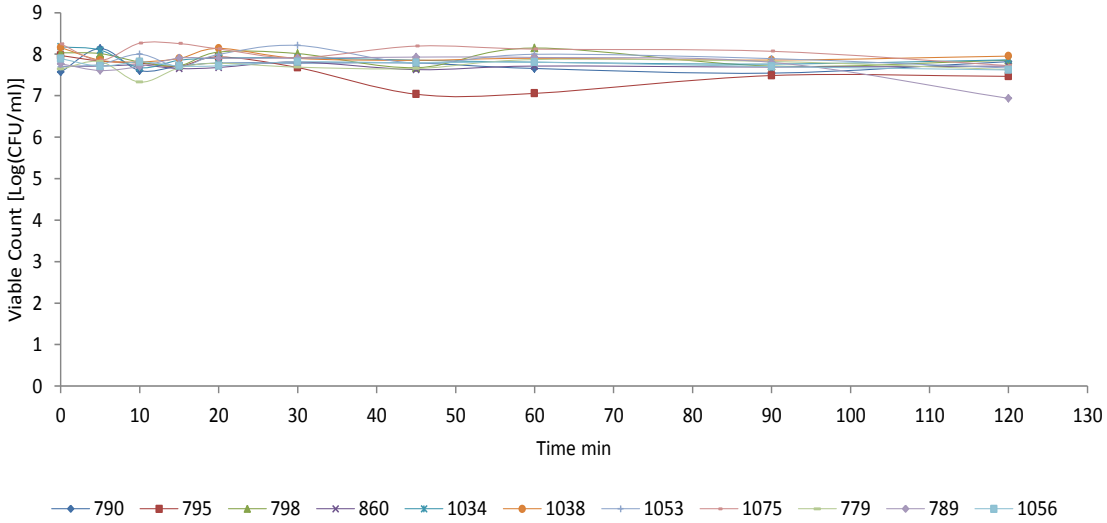


Figure 4.12: Survival of *Ent. hormaechei*, *Ent. cloacae* and *Ent. aerogenes* strains in casein-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

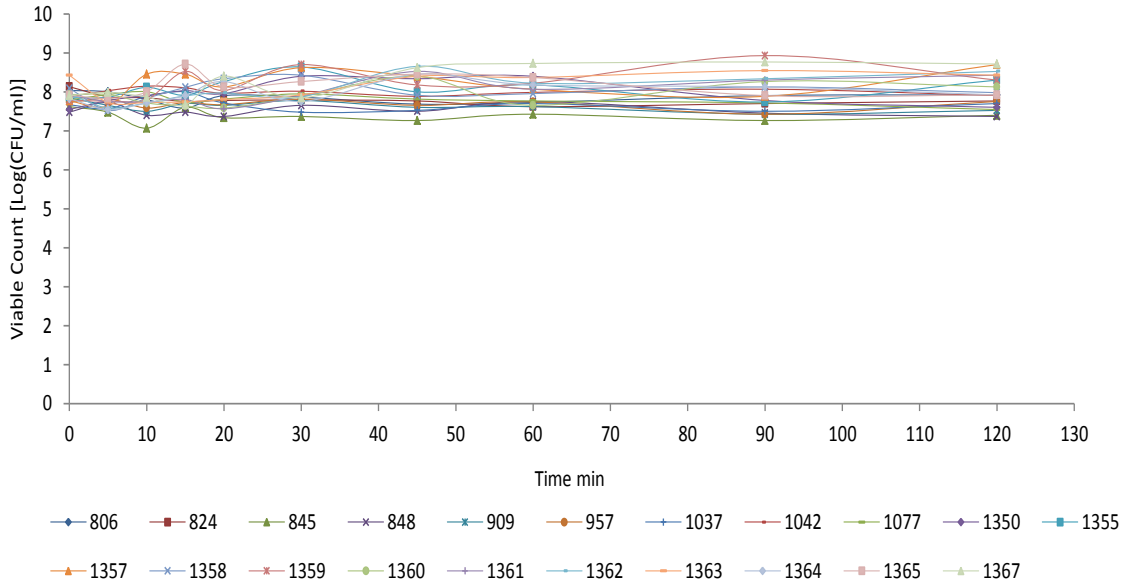


Figure 4.13: Survival of *Ent. cancerogenus* in whey-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

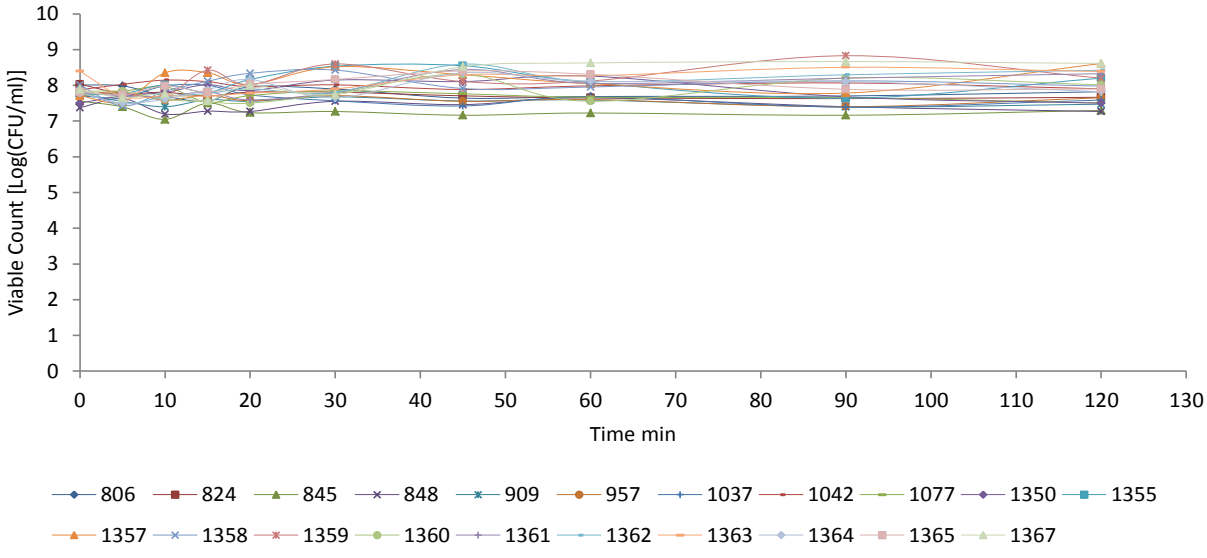


Figure 4.14: Survival of *Ent. cancerogenus* in casein-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

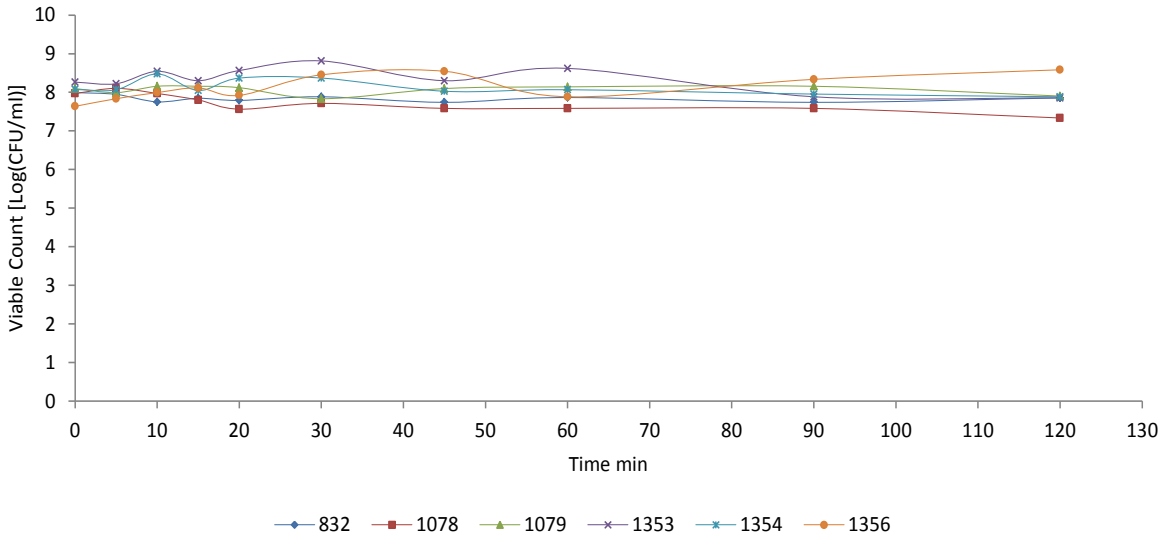


Figure 4.15: Survival of *K. oxytoca* in whey-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

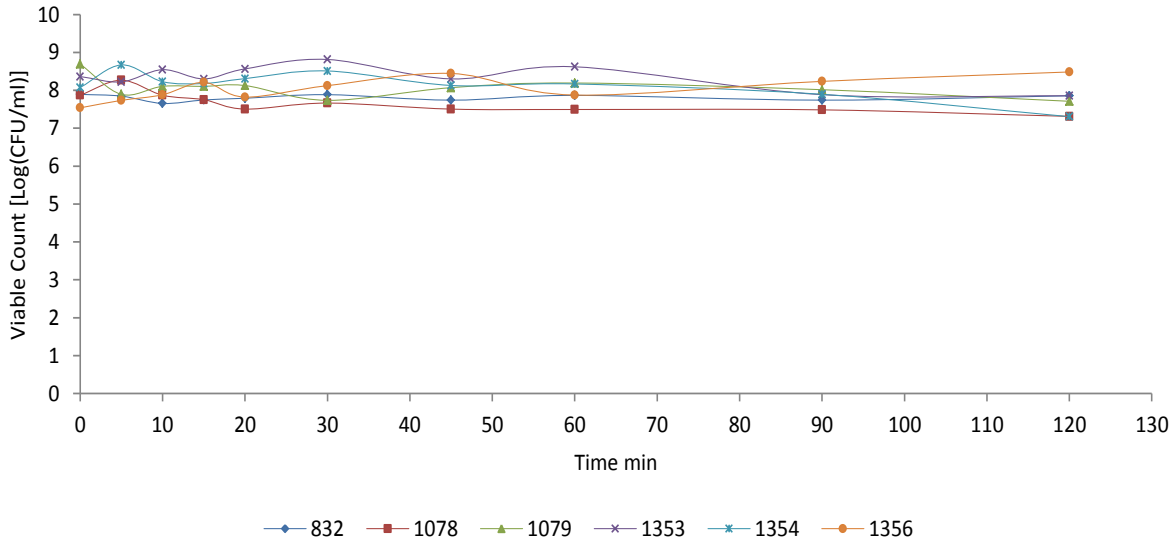


Figure 4.16: Survival of *K. oxytoca* in casein-based infant formula at pH 3.5 for 2h.

Experiments were performed in duplicate and data are presented as the average of two independent experiments. The viable count of cells was measured during 2h at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes.

4.4 DISCUSSION

The present study was designed to determine the physiological attributes for representative strains defined by using PFGE, with respect to stress survival which was affected by formula type and temperature at 55°C, biofilm formation, capsule production and acid tolerance of organisms to pH 3.5 (HCl acidified formula).

Most strains survived at 55°C (Tables 4.1 and 4.2). In this study, the considerable finding was that same death rate at 55ºC was demonstrated by strains which belonged to the same cluster by PFGE analysis. For example, the pulsotypes of Enc1, Enc2, Enc3, Ebh6 and Kc3 *Ent*. *cancerogenus* strains 845, 848, 1355, 1358, 1363 and 1364, *Ent. hormaechei* strains 795 and 798, and *K. oxytoca* strains 1078 and 1079, respectively were sensitive to heat tolerance at 55ºC on whey and casein-based infant formula.

This finding may suggest that these strains was possibly originated from the same source and the same clone contaminated different feeding tubes. For the MBM strains *Ent*. *cancerogenus* strains (1355, 1358, 1363 and 1364), it was probably that these organisms were isolated from the same mother. Therefore, the neonates could be exposed to the ingestion of these organisms either from contaminated milk or from infant formula. The findings of this study were consistent with Forsythe (2009), who found that in a neonatal care unit, fortified breast milk and reconstituted powdered infant formula were reconstituted at room temperature when required, and not at higher temperatures. Similar procedures were reported by Rosset *et al.* (2007) in a study of French hospitals in that samples were reconstituted with room temperature water.

On the other hand, the ability of tested strains to survive at 55ºC may become a sourse of infection of neonates in NICUs which was previously described by Iversen *et al*. (2004a). They found that *C. sakazakii* is capable of growing in PIF during storage at refrigeration temperatures and adhere to infant-feeding equipment, which probably becomes a sourse of infection. In addition, *C. sakazakii* strains were able to grow in PIF between 6° and 45°C, with the optimum being 37° to 43°C (Iversen *et al*., 2004a). The meeting by FAO and WHO in 2006 acknowledged that the reconstitution of PIF with water between 40° and 50°C (unless used immediately),

holding bottles at room temperature and extended feeding periods were linked with a rise in relative risk.

Biofilm formation was investigated as this may be a means of organism persistence in the NICUs and attachment to enteral feeding tubes. This study found that the relative biofilm formation was higher at 37ºC compared with 20ºC by most tested strains in all types of formula (Figures 4.9, 4.10 and 4.11). The notable result was that the *Ent. hormaechei* strains 1053 and 1075 and *Ent. cancerogenus* strains 1355, 1358, 1359, 1360, 1363 and 1364 were clustered into 4 pulsotypes, Ebh3, Enc2, Enc1 and Enc3 (each pulsotype consists of two strains) respectively, and formed the same quantity of biofilm in whey and casein-based infant formula at 37ºC and 20ºC. However, *Ent. hormaechei* strains 1053 and 1075 only exhibited the same level of biofilm in whey-based infant formula at 37ºC (Table 4.3). This finding probably indicates that the same clone was cross contaminated between different feeding tubes and the prevalence of same clone was recovered from the same mother (MBM strains).

The quantity of biofilm that was detected on the three types of formula was higher in casein-based infant formula compared with whey and soya-based infant formula in all strains. These observations indicate the importance of the nutrient type present in milk in biofilm formation. Furthermore, these findings are in agreement with other studies (Kim *et al.,* 2006; Meadows, 1971). Research has shown that nutrients and other media components influence the attachment of bacteria to surfaces of various materials (i.e biofilm formation) (Hood and Zottola, 1997). Meadows (1971) found that casein enhanced the attachment of *E. coli*, *P. fluorescens* and *Aeromonas liquefaciens* to glass surfaces. Whey protein has shown the same effect in stainless steel surfaces (Speers *et al.,* 1985).

In general, across all the bacterial species that were tested, this study confirmed the previous research (Hurrell *et al*., 2009) which demonstrated that the organisms may originate from the attached bacterial biofilm, and break off in clumps. The bacterial multiplication occurs at room temperature during the feeding period compared with the tube at 37°C (which can be in place for >48 hours). This can result in the contamination of fresh feed in the tube lumen leading to further bacterial multiplication. Bacteria enter the neonate's stomach as clumps of cells which may

survive passage through the neonate's stomach. The ability to invade intestinal cells may be a strong indication of potential virulence and poor outcomes in the neonatal host.

The ability of strains to produce capsular material was determined by colony appearance on milk agar (whey, casein and soya) at 20°C (after 24 hours and 48 hours) and 37°C (after 24 hours). The major of *Ent. ludwigii*, *Ent. cloacae*, *Ent. aerogenes*, *Ent. hormaechei*, *Ent. cancerogenus* and *K. oxytoca* strains demonstrated ability to produce capsular material on the three types of milk agar (whey, casein and soya) at 37ºC compared with 20ºC. A possible explanation for this might be that body temperature possibly plays an important role in the indication of capsule material.

In this study, an interesting finding is that all species are able to produce capsule material in soya-based infant formula compared with whey and casein-based infant formula at 37ºC (Tables 4.3 and 4.4). This finding may be a strong indication that the differentiation of composition between the three types of formula possibly affects the strains produce capsular material. For example, whey and casein-based infant formula is comprised of lactose as the carbohydrate source while soya-based infant formula consist of glucose which may be required for the bacteria to produce capsular material.

A further discovery was the correlation between biofilm formation and capsular material production on casein milk agar at 20ºC and 37ºC by all species except *Ent. hormaechei*. This could be due to the casein-based infant formula nutrients required to get attached to the surface, such as a feeding tube, to survive and grow. These observations are consistent with Van Acker *et al.* (2001) who suggested that infant formula milk may contain nutrients which were important for bacteria (*C. sakazakii*) for pre-attachment to stainless steel or feeding tubes to survive and grow.

In addition, Scheepe-Leberkühne and Wagner (1986) also suggested bacterial attachment and adherence to a surface can be enhanced by producing exopolysaccharide capsular material. Another previous study also stated that profuse capsular material may contribute to forming biofilm by clinical isolates of *Cronobacter* (Caubilla-Barron *et al*., 2007).

Tolerance of acidic conditions was determined by expository tested strains to two types of formula (whey and casein) after adjusting the pH 3.5 for two hours at body

temperature (Figures 4.12-4.19). All of the tested strains were able to tolerate the 2 hours exposure time at pH 3.5. The viable counts were no difference between the two types of infant formula.

The results of this research support the idea that gastric juice is playing an important role in controlling the outcome of food-borne infections. The capability of organisms to survive in the acidic environment of the stomach is an indictor that these organisms may be pathogenic bacteria which could be able to invade the intestine, thus causing human illness. Previous studies found that the reduction in gastric acidity promotes survival rates of some common food-borne pathogens (Peterson, 1989) and with a reduction of the infective dose (Cash *et al.,* 1974; Schlech *et al.*, 1993).

Chapter 5: VIRULENCE FACTORS

5.1 INTRODUCTION

The ability of bacterial strains to cause infection depends upon a number of factors including (but not limited to) adhesion, colonisation, activation of disease process, serum resistance and the production of toxins. However, the essential factor is the capability of the micro-organism to attach to the host surfaces, such as mucous membranes, gastric and intestinal epithelial or endothelial tissue (Boyle *et al.*, 2003; Finlay and Falkow, 1997; Klemm and Schembri, 2000). There is significant evidence which indicates that markedly diverse microbial pathogens utilize common strategies to cause infection and disease. For example, numerous different bacterial pathogens share common mechanisms in expression of their abilities to attach, invade and cause damage to host cells and tissues, in addition to surviving host defences, thereby causing infection (Wilson *et al.*, 2002).

In order to study the host, pathogens and intestinal micro-flora interactions tumour derived models such as Caco-2, T84 and HT-29 are widely used (Cencič and Langerholc, 2010). Caco-2 cell lines are used as the model cell lines for the small intestine while HT-29 for the large intestine *in vitro* to determine the ability of bacterial adhesion and invasion to human cells and tissues (Grajek and Olejnik, 2004). Enterotoxigenic *E. coli* is one example causing diarrhoea by secretion of enterotoxin after attaching to the intestinal mucosa (Gaastra, 1996).

The host immune response utilizes phagocytic cells as a critical line of defence against infection. The capability of a pathogen to persist and replicate within macrophages is a potent way of evading the defence mechanisms of the host. *Sal.* Typhimurium is one of the examples which enters and replicates within the macrophages and causes damage to them (Lindgren *et al.*, 1996). *C. koseri* causing neonatal infection is another bacterium that displays the ability to survive and replicate within macrophages which in turn may contribute to the establishment of chronic central nervous system infections including brain abscesses (Townsend *et al.*, 2003). A further study by Townsend *et al.* (2008a) found that *Cronobacter* spp. strains showed the ability to persist and even replicate within human U937 macrophages.

Despite the availability of effective antimicrobial therapy, neonatal bacterial meningitis remains a disease with unacceptable rates of morbidity and mortality. Community-acquired bacterial meningitis in Europe and the United States has a strong association with *Se. pneumoniae* and *Neisseria meningitidis*, both of which are considered as the most common etiologic agents; *Se. pneumoniae* alone is associated with two-thirds of cases (Arda *et al*., 2008; Brouwer *et al*., 2010; van de Beek *et al*., 2004). Additionally, fatal infections of neonates, such as necrotizing enterocolitis, septicaemia and meningitis, have also been associated with *Cronobacter* (Bowen and Braden, 2006 and 2008).

The blood-brain barrier model of HBMEC is used to study the interaction between pathogenic bacterial and HBMEC *in vitro*. The blood-brain barrier is responsible for preserving biochemical homeostasis within the central nervous system (CNS). It occurs as a single layer of specialised brain microvascular endothelial cells (BMEC) which display continuous tight junctions and a conspicuous absence of pinocytosis (Betz and Goldstein, 1986; Patrick *et al.,* 1992).

A study by Badger *et al.* (1999) demonstrated that *Citrobacter* has shown the ability to invade and replicate within HBMEC. Histological studies of infant rat brains infected with *C. koseri* had shown that this model closely mimics the course of infection observed in humans (Kline *et al.,* 1988). Previous studies have shown that *E. coli* K1 and group B streptococci (GBS) invaded brain microvascular endothelial cells (BMEC) *in vitro* and were able to penetrate the blood brain barrier in the experimental newborn rat model of haematogenous meningitis (Badger *et al.,* 1998; Kim *et al*., 1992; Nizet *et al.,* 1997).

Similarly, *C. sakazakii* strains isolated from a NICU outbreak were able to invade rBCEC4 (Townsend *et al*., 2008a).Virulence traits were investigated also for *Ent. hormaechei* strains by Townsend *et al*. (2008b), who demonstrated that *Ent. hormaechei* strains were shown to invade both gut epithelial (Caco-2) and blood–brain barrier endothelial cells (rBCEC4), and to persist in macrophages (U937).

The most common feature of the invasive bacterium is its ability to avoid the bactericidal effects of host serum (Hoe *et al*., 1999; Hol *et al.,* 1995; Porto *et al.,* 1989). Briefly, a bacterium that has the ability to survive on human blood possesses the potential to spread to different organs, avoiding the killing mechanism of

complement and antibody-mediated opsonization. In addition, serum resistance in species comprising of pathogens and commensals is often attributed to the pathogens as an acquired trait that allow them to cause disease in their host (Williams *et al.*, 2001).

Specifically, serum resistance is a common trait of meningococci isolated from blood or cerebrospinal fluid (Figueroa and Densen, 1991). Clinical isolates of *V. vulnificus* were able to survive in human serum (Bogard and Oliver, 2007). Furthermore, a previous study by Townsend *et al*. (2008b) reported that *Ent. hormaechei* strains isolated from different neonates during a nosocomial outbreak in a California hospital were serum-resistant.

The production of haemolysis is also associated with virulence in different bacterial species. Haemolysins produce a group of lytic toxins which then play an essential role in the disease production. For example, *E. coli* produces α- or β haemolysins. These two types of haemolysin are required for the exponential growth, but the calcium ions are required for lytic activity by α- haemolysins (Chart *et al.*, 1998). β haemolytic group A streptococi and *S. aureus* break down the barriers of a tissue to spread in the host while the microbes remain outside the host cells: this strategy is called extracellular invasion. These species are able to secrete some enzymes that degrade host cell molecules such as hyaluronidase (cleaves proteoglycans in connective tissue), streptokinase and staphylokinase (breaks down fibrin clots), lipase (degrades accumulated host oils), and nuclease (digests released RNA and DNA) (Wilson *et al.*, 2002).

Iron is an essential element of bacterial pathogenesis for growth, replication and progression of infection (Braun and Winkelman, 1987). The ability of pathogenic bacteria to obtain iron from the host is one of the determinants for bacterial virulence. Bacteria require iron for a variety of metabolic processes, such as amino acid synthesis, oxygen transport, respiration, nitrogen fixation, methanogenesis, the citric acid cycle, photosynthesis and DNA biosynthesis, and the amount of free iron available in the human host is not enough for bacterial growth because of the presence of iron-binding glycoproteins such as transferrin and lactoferrin (Sandy and Butler, 2009).

The microorganisms act to synthesize and secrete siderophores, low-molecular-weight, affinities for ferric iron, which act to sequester and solubilise the iron from host sources and facilitate its uptake by the bacterium (Pal and Karuna, 2010; Schwyn and Neilands, 1987; Crosa, 1987). Yersiniabactin is a part of an iron-uptake system which has been first described in *Yersinia* spp., and the genes are located on a High-Pathogenicity Island (HPI) (Carniel, 2002). Lately, the existence of yersiniabactin-mediated iron uptake system has been discovered in several species of the family *Enterobacteriaceae* (Bach *et al*., 2000; Schubert *et al.,* 2000). Previously the studies have suggested that *Yersinia* spp., *E. coli* and probably *K. Pneumonia* isolated including the HPI are more virulent than strains lacking this island (Bearden *et al*., 1997; Carniel *et al*., 1992; Lin *et al*., 2008).

The HPI of *Y. pestis* is known as the *pgm* locus, which contains about 102 kb of chromosomal DNA and comprises of the *irp1* and *irp2* and *fyuA* or *psn* genes involved in iron storage and uptake the hemein storage (hms) locus (Perry *et al*., 1990). The *irp1* and *irp2* genes coding for the two iron-repressible high-molecular-weight proteins HMWP1 and HMWP2, which seemingly are involved in the production of yersiniabactin (Lucier *et al*., 1996); and the *fyuA* or *psn* gene plays an important role for ferric yersiniabactin uptake or pesticin sensitivity where it serves as coding for the yersiniabactin receptor. *FyuA* also acts as a receptor for pesticin (Carniel *et al.,* 1992; Carniel *et al.,* 1989; Heesemann *et al.,*1993; Rakin *et al.,*1994).

In Gram-negative pathogens, the most important contributing factor to β-lactam resistance is β-lactamase production (Livermore, 2003). β-lactams are various antibiotic molecules that are categorised based on their chemical structures (Pitout and Laupland, 2008). The β-Lactam family of molecules is constituted of four groups: penicillins, cephalosporins, monobactam, and carbapenems. Carbapenems (imipenem, ertapenem, meropenem, and doripenem) are the most recently developed molecules having a broad spectrum of antibacterial activity (Nordmann *et al.,* 2012), While β lactamases are enzymes produced by bacteria that inactivate β-lactam antibiotics by hydrolysis.

These enzymes are divided into four groups depending on their activity profile. Description of these enzymes is given below:

(i) Penicillinases inactivate penicillins, but do not affect cephalosporins, aztreonam, or carbapenems;

(ii) Cephalosporinases preferentially inactivate cephalosporins and aminopenicillins, but do not degrade other penicillins (carboxy- and ureido-penicillins), aztreonam, and carbapenems;

(iii) Extended-spectrum β-lactamases (ESBLs) act to inactivate all β-lactams except carbapenems and are inhibited by clavulanic acid; and

(iiii) Carbapenemases deactivate carbapenems and may also deactivate other types of β-lactam molecules, depending on the enzyme (Nordmann *et al*., 2012).

The ESBLs are one group of β lactamases which have the capability to hydrolyse and cause resistance to different types of the newer β-lactam antibiotics, comprising the expanded-spectrum (or third-generation) cephalosporins (eg, cefotaxime, ceftriaxone, ceftazidime) and monobactams (eg, aztreonam), but not the cephamycins (eg, cefoxitin and cefotetan) and carbapenems (eg, imipenem, meropenem, and ertapenem) (Bradford, 2001).

The ability of organisms to produce ESBLs remains an important reason for therapy failure with cephalosporins and possess major consequences for infection control (Paterson and Bonomo, 2005). Furthermore, most of ESBLs can be divided into three groups: TEM, SHV, and CTX-M types (Paterson and Bonomo, 2005). The major ESBLs-producing organisms isolated worldwide are *K. pneumoniae* and *E. coli*, but these enzymes have also been identified in some other members of *Enterobacteriaceae* and in particular non-fermenters (Jacoby and Munoz-Price, 2005). In a Further discovery by Hurrell *et al*. (2009b), the antibiograms for the *Enterobacteriaceae* were isolated where all the *Se. marcescens* strains were resistant to amoxillin and co-amoxiclav. In addition, a quarter of *Ent. hormaechei* isolates were resistant to the 3rd generation cephalosporins, ceftazidime and cefotaxime. During the period of study, *K. pneumoniae* and *Se. marcescens* caused infections in the two NICUs.

The ESBLs were detected in three of these strains (Hurrell *et al*., 2009b). The antibiotic resistance patterns of the remaining strains could be due to repressed chromosomal AMPC β-lactamase production (Bonnet, 2004). It was proposed that

the empirical use of antibiotics may be selecting for ESBL organisms such as *Se. marcescens* and *K. pneumoniae*. Although no link was established (due to restrictions in sampling) with feeding tube isolates, it was notable that these two species were also responsible for neonatal infections in both NICUs during the study period.

Bacteria are known to attach and grow in feeding tubes, but further details regarding their potential virulence is still awaited. Hence, this part of the project was focused on the virulence potential of *Ent. ludwigii, Ent. hormaechei, Ent. cloacae, Ent. aerogenes* and *K .oxytoca*.

5.2 Materials and methods

Thirty four *Enterobacteriaceae* composed of *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae, Ent. cancerogenus* and *K. oxytoca* were isolated from both the previous Nottingham NICU study (Hurrell *et el.*, 2009b) and from Delgado *et al.* (2008). They were used to determine the ability of the strains to attach to the human epithelial cells, as given in Tables 2.4 and 2.6. The attachment and invasion of Caco-2 human epithelial cells was determined after a 3 hours exposure period. *E. coli* 1230 and *Salm.* Enteritidis strain 358 were used as negative and positive controls respectively. *Salm.* Enteritidis is a documented enteric pathogen capable of attaching and invading Caco-2 cells, the procedure described in section 2.5.1.3 and 2.5.1.4.

Selected strains listed in Table 2.7 section 2.1.1 in chapter 2, were used in this study. As *Ent. hormaechei* is associated with cases of bacterial meningitis and also we have *Ent. ludwigii* strain (NTU1439) from Nottingham QMC hospital that was isolated from a case of neonatal meningitis during the period of study, thus we carried out an *in vitro* study to investigate the ability of these strains to invade capillary endothelial cells which comprise the HBMEC the procedure described in section 2.5.1.3 and 2.5.1.4. *Ent. aerogenes* and *Ent. cloacae* were not included in this study because, at the time this study was initiated, these strains were considered as *K. oxytoca*.

However, later we identified these strains as *Ent. aerogenes* and *Ent. cloacae* using 16S rDNA sequence analysis. The attachment and invasion of HBMEC was determined after a 3 hours exposure period the procedure described in section 2.5.1.3 and 2.5.1.4. *E. coli* 1230 and *C. koseri* strain 48 were used as negative and positive

controls respectively. The *in vitro* study we carried out to determine the ability of *Ent. ludwigii*, and *Ent. hormaechei* to invade rBCEC4 was compared with the HBMEC cell line. The positive and negative controls were *C. koseri* 48 and *E. coli* 1230 respectively.

The U937 macrophage cell lines were used to investigate the ability of tested strains to persist in macrophages for up to 48 hours. *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cloacae*, *Ent. aerogenes* and *K. oxytoca* strains were used to infect the U937 macrophage cell line. These strains were used with previous cell lines. Strains of *C. koseri* 48 and *E. coli* 1230 were used as positive and negative controls respectively; section 2.5.1.5. Giemsa staining was used to stain Caco-2 human epithelial cells after 3 hours incubation of ~10^8 bacteria. As part of a study, the virulence factor, haemolysin production, was examined and the method was described in section 2.5.2.

The ability of bacteria to resist serum was detected because serum resistance is attributed to disease in their host. The method is described in section 2.5.3. Siderophore production was carried out to determine the ability of strains to obtain iron from the host. The method is described in section 2.5.4. The antimicrobial susceptibility of each strain to several classes of antibiotics was tested by using the disc diffusion method. As recommended by Health Protection Agency (2006), *E. coli* NCTC 10418 was used as the control strain. A PCR detection analysis was used to determine the high pathogenicity island genes. The preparations of culture media and conditions for this chapter were described previously in section 2.5 in chapter 2 of materials and methods.

5.3 Results

Bacterial attachment and invasion of host cells

The tested bacteria strains showed interacted of bacteria with different mammalian cells and the results are given in the following section:

5.3.1 Bacterial attachment to Caco-2 human epithelial cells

The Caco-2 cell line was used to determine the ability of the strains to attach to the human epithelial cells. To facilitate the classification of strains, attachment levels were divided into three groups (high, medium and low) the cut of point. The cut off points were experimental decision because of the variation between results of strains.

A cut off value of more than 10% of recovered cfu/ml was considered as the high attachment of strains to Caco-2 human epithelial cells, medium attachment refers to the attachment of strains between 5% and 10% cfu/ml, while less than 5% cfu/ml was considered as low attachment.

As seen in Figure 5.1, all strains showed the ability to attach to the Caco-2 human epithelial cells. Two of the *K. oxytoca* strains, 1356 and 1079, showed a high level of attachment. A medium level of attachment was shown by 4/6 strains of *Ent. ludwigii*, 3/8 strains of *Ent. hormaechei* and 1/6 strains of *K. oxytoca*, whereas low attachment was reported by 2/10 strains of *Ent. ludwigii*, 5/8 strains of *Ent. hormaechei*, 3/6 strains of *K. oxytoca* and all *Ent. aerogenes* and *Ent. cloacae* strains,

As shown in Figure 5.2, all *Ent. cancerogenus* strains attached to Caco-2 human epithelial cells and the high level of attachment was shown by strains 1350, 1077, 1037, 806 and 1360, whereas the medium level of attachment was observed by strains 845, 957, 1359, 1355 and 1364. The rest of *Ent. cancerogenus* strains showed low attachment to Caco-2 human epithelial cells. Using one-way ANOVA, *Ent. Ludwigii*; strains 1348, 1349, 1351 and 1366, *Ent. hormaechei* strains; 798, 860 and 1034,
Ent. cancerogenus strains; 806, 845, 957, 1037, 1077, 1350, 1355, 1359, 1360 and 1364 and *k. oxytoca* strains; 1353, 1356 and 1079 showed significantly more attachment than *E. coli* 1230 ($p < 0.05$).

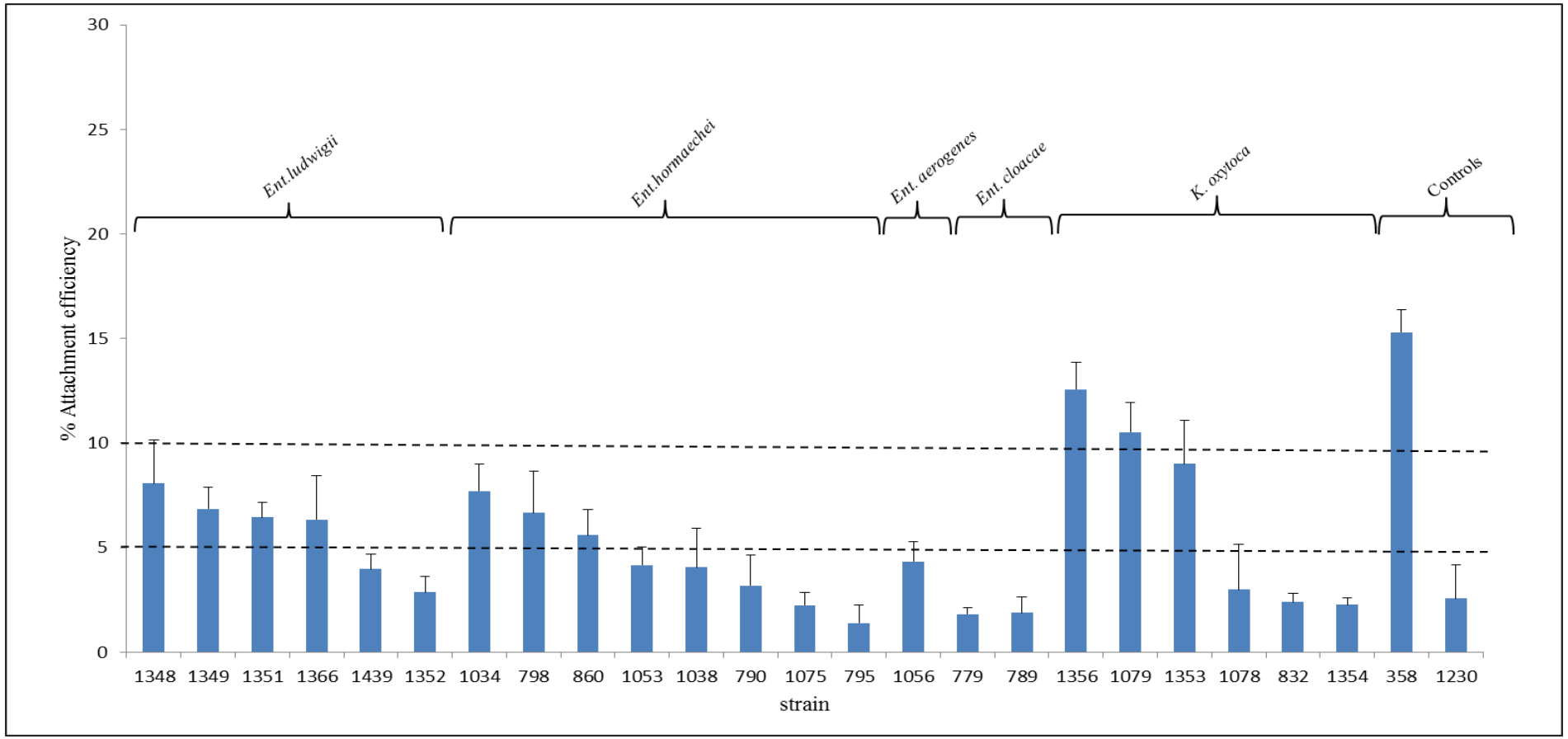


Figure 5.1: Attachment of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. aerogenes*, *Ent. cloacae* and *K. oxytoca* to the mammalian Caco-2 cell line. *Salm.* Enteritidis 358 and *E. coli* 1230 were used as positive and negative controls respectively. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments. MOI= 100.

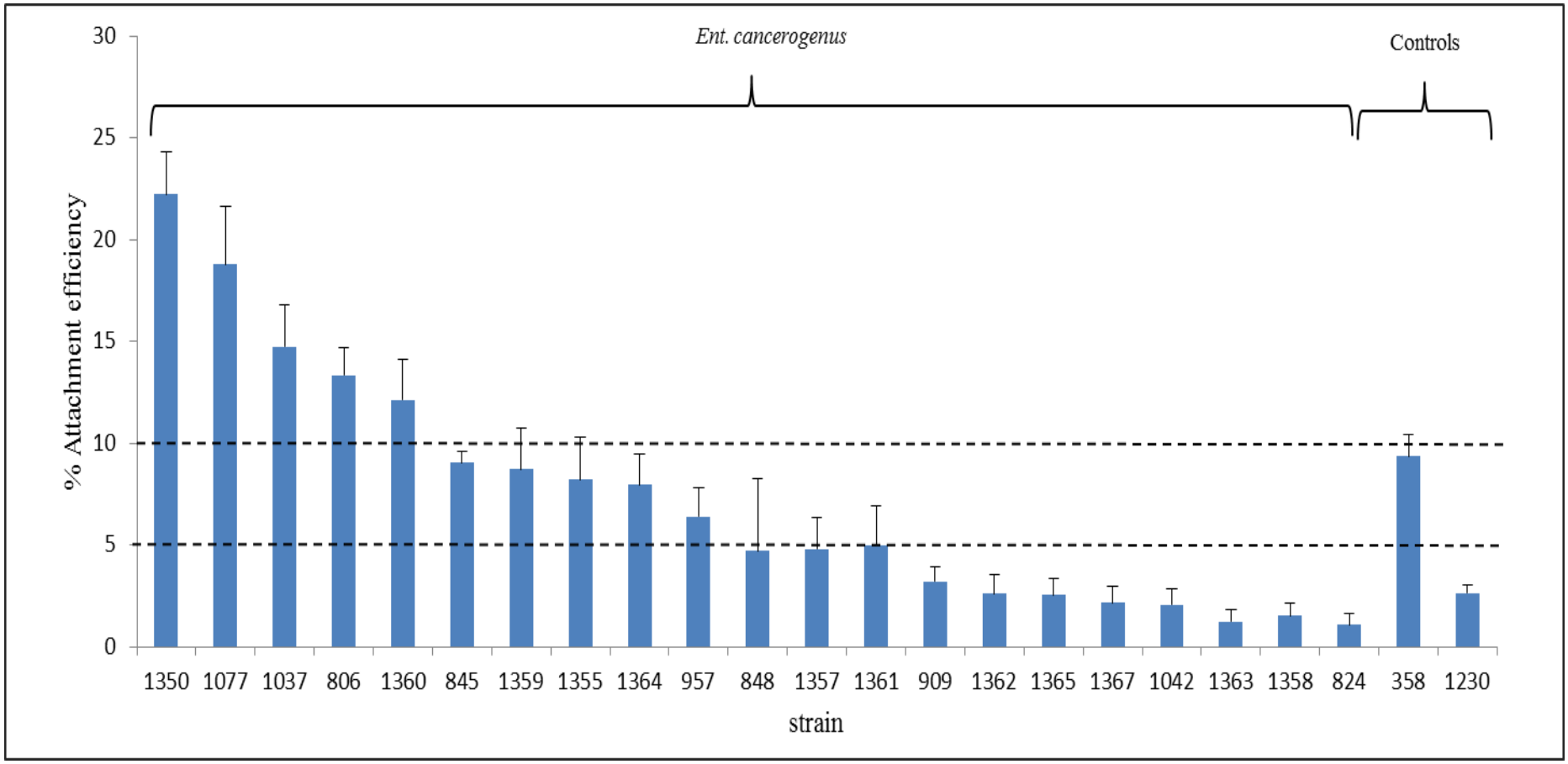


Figure 5.2: Attachment of *Ent. cancerogenus* to the mammalian Caco-2 cell line.

Salm. Enteritidis 358 and *E. coli* 1230 were used as positive and negative controls respectively. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments. MOI= 100.

5.3.2 Bacterial invasion Caco-2 human epithelial cells

To determine the ability of strains to invade Caco-2 human epithelial cells, gentamicin protection assay was used. In Figures 5.3 and 5.4 most strains invaded Caco-2 human epithelial cells at very low levels, however *Ent. hormaechei* strain 1053 showed four fold less invasion to Caco-2 human epithelial cells than *Salm.* Enteritidis.

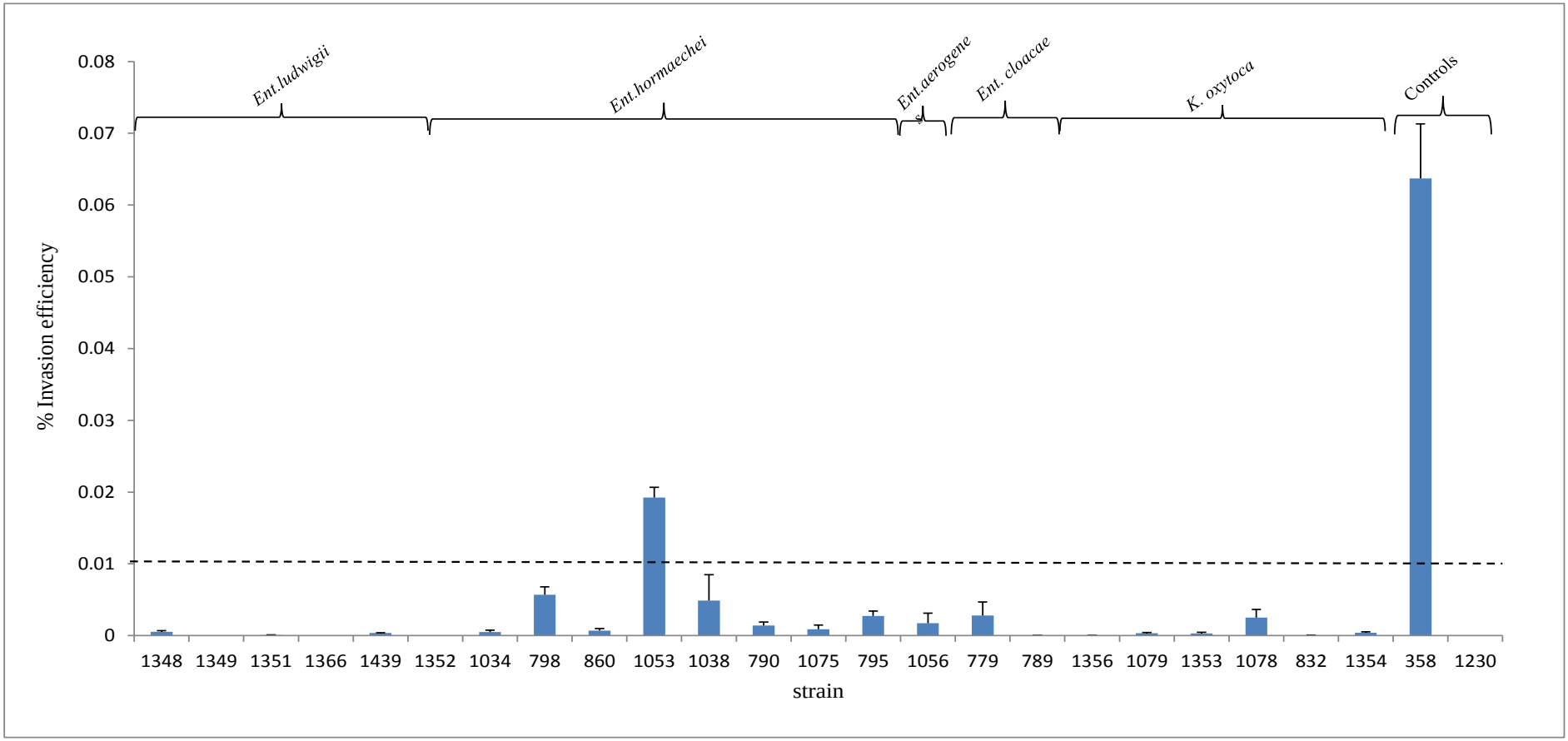


Figure 5.3: Invasion of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. aerogenes*, *Ent. cloacae* and *K. oxytoca* to the mammalian Caco-2 cell line. *Salm.* Enteritidis 358 and *E. coli* 1230 were used as positive and negative controls respectively. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments. MOI= 100.

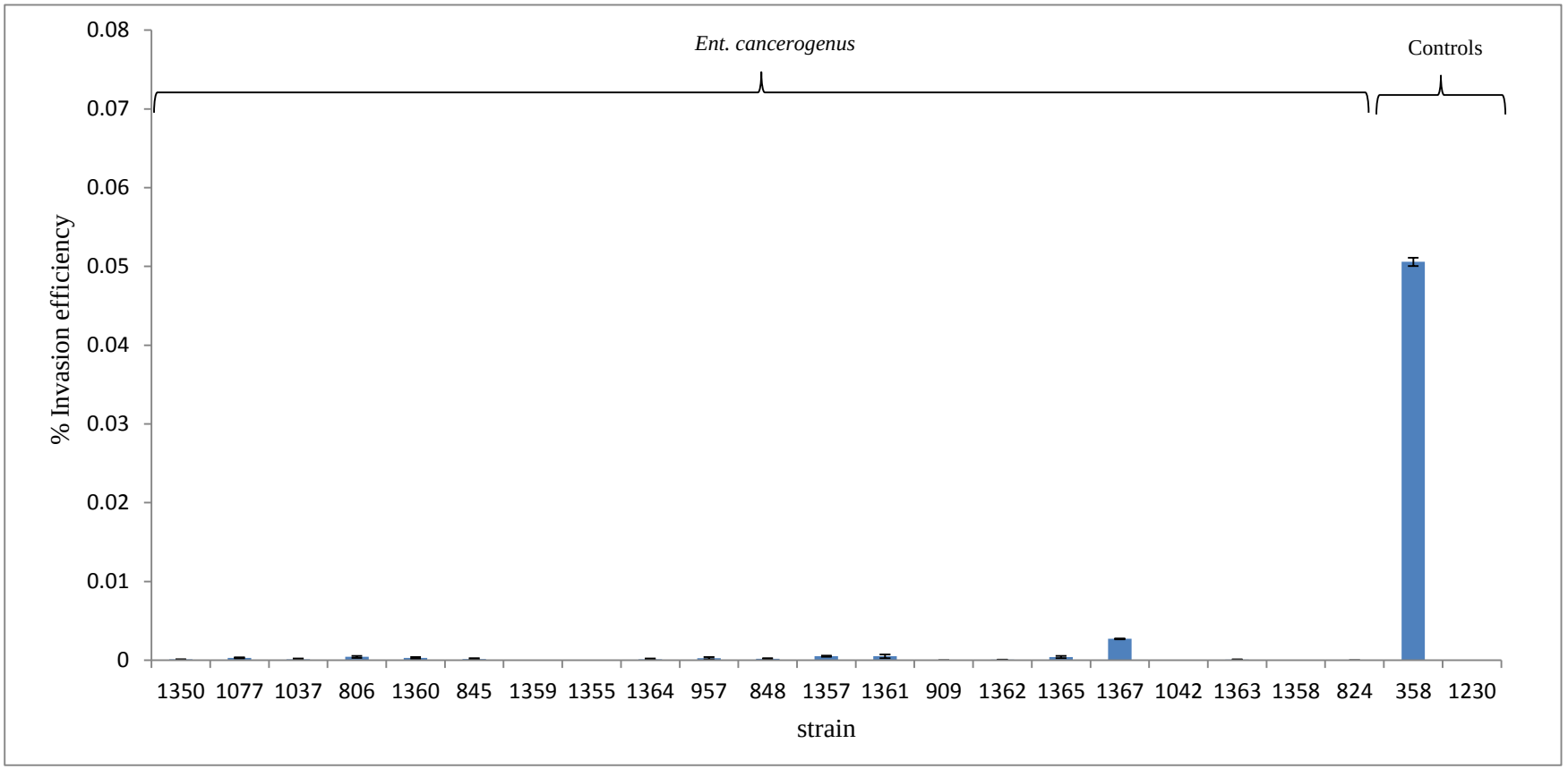


Figure 5.4: Invasion of *Ent. cancerogenus* the mammalian Caco-2 cell line.

Salm. Enteritidis 358 and *E. coli* 1230 were used as positive and negative controls respectively. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments. MOI= 100.

5.3.3 Bacterial attachment HBMEC

The HBMEC cell line was used to determine the ability of the strains to attach to the HBMEC**.** The attachment levels were divided into three groups (ie high, medium and low). Since it was a variation between results of strains, therefor the cut off points were experimental decision. The cut off values to define the groups are given below:

1. High attachment level= >6% cfu/ml
2. Medium attachment level= 2-6% cfu/ml
3. Low attachment level= < 2% cfu/ml.

Figure 5.5 illustrates that all strains showed the ability to attach the HBMEC and the rate of attachment was less than 6% of recovered cfu/ml. Three of *Ent*. *ludwigii* strains 1366, 1351 and 1348 and 6/8 of *Ent. hormaechei* showed medium levels of attachment, whereas low attachment was reported by the rest of the strains. The positive control showed a high level of attachment that was 12% of recovered cfu/ml. Statistically, one of *Ent. ludwigii* strain 1366, five out of eight *Ent. hormaechei* strains 795, 798, 1034 1038 and 1053 were showed significantly more attachment than *E. coli* 1230 ($p < 0.05$).

5.3.4 Bacterial invasion of HBMEC

To detect the value of invasion of HBMEC cells by tested strains, the level of invasion was divided into two categories:

1. < 0.1% cfu/ml low value of invasion
2. > 0.1% cfu/ml high value of invasion

As shown in Figure 5.6, there was no invasion by most of *Ent. ludwigii* except strain 1439 which invaded at a very low rate of recovered cfu/ml, while 4/8 of *Ent. hormaechei* invaded HBMEC and the high invasion was by strain 1075.Thus, on average, *Ent. hormaechei* was the most invasive as compared to *Ent. ludwigii*.

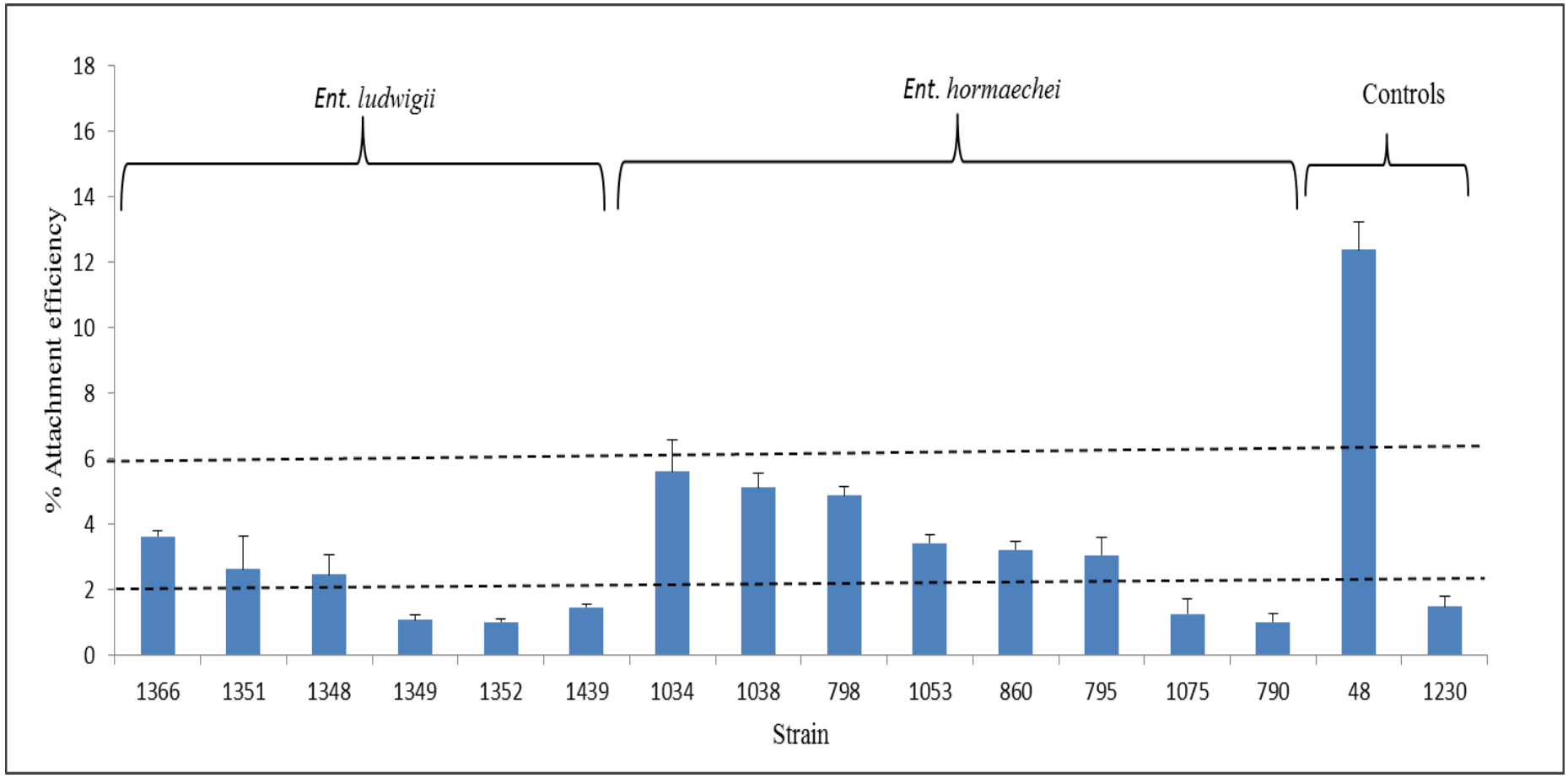


Figure 5.5: Attachment of *Ent. ludwigii* and *Ent. hormaechei* to the mammalian HBMEC cell line.

E. coli 1230 and *C. koseri* 48 were used as negative and positive controls. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments.

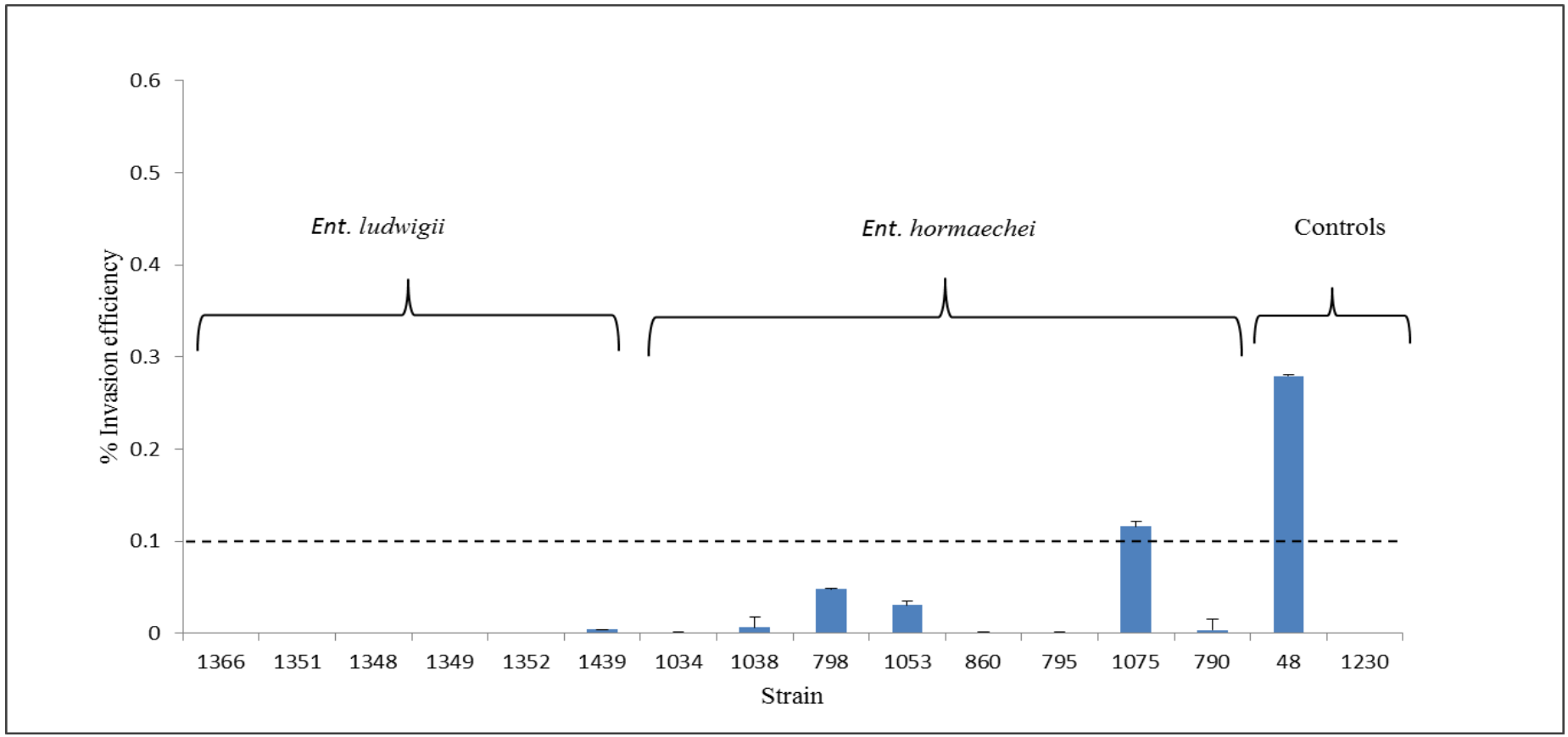


Figure 5.6: Invasion of *Ent. ludwigii* and *Ent. hormaechei* to the mammalian HBMEC cell line.

E. coli 1230 and *C. koseri* 48 were used as negative and positive controls. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments.

5.3.5 Bacterial attachment rBCEC4

Figure 5.7 shows a range of attachment of *Ent. ludwigii,* and *Ent. hormaechei* to rBCEC4. The cut-off to define the degree of attachment is given below:

1. High attachement level = > 6% cfu/ml
2. Medium attachment level = 2-6% cfu/ml
3. Low attachment level = < 2% cfu/ml

Ent. ludwigii strains 1348, 1366 and 1351 and *Ent. hormaechei* strains 860, 1075, 795, 1038 and 1053 showed high levels of attachment where the number of recovered cells was in the range between 9.50% to 14.50% cfu/ml. The medium level of attachment was by 2/6 of *Ent. ludwigii* and 1/8 of *Ent. hormaechei,* while low attachment was shown by the rest of the strains.

5.3.6 Bacterial invasion of rBCEC4

The ability of tested strains to invade the rBCEC4 was determined. The level of invasion was divided into two groups high and low. The degree of invasion is defined as is given in section 5.3.4 (see Figure 5.8).

All *Ent. ludwigii* and *Ent. hormaechei* invaded rBCEC4 at different values. One strain of *Ent. ludwigii,* which was NTU1439 from Nottingham QMC hospital from a case of neonatal meningitis, and 3/8 strains of *Ent. hormaechei* (798, 1053 and 1075) showed high levels of invasion. The value recovered cells were between 0.15% to 0.50 % cfu/ml. The rest of strains invaded the rBCEC4 at a low level, which were less than 0.1% cfu/ml. *C. koseri* strain 48 (positive control) invaded rBCEC4 at 0.35% cfu/ml.

Statistically, All *Ent.ludwagii* strains except strain 1349 and *Ent. hormaechei* strains 860, 795, 1038, 1053 and 1075 attached rBCEC4 cells at a significantly ($p \leq 0.05$) greater value than *E. coli* 1230.

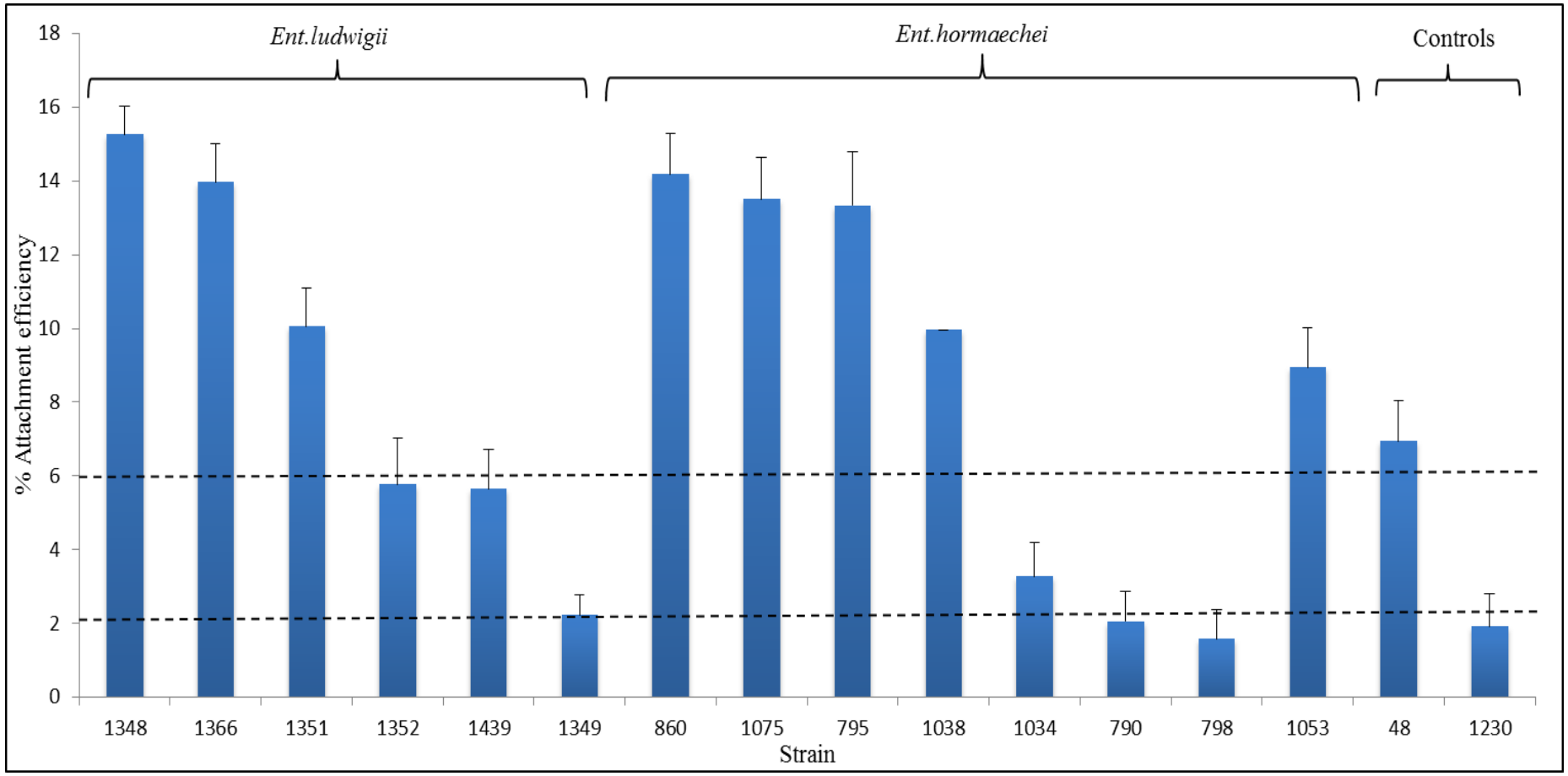


Figure 5.7: Attachment of *Ent. ludwigii* and *Ent. hormaechei* to the mammalian rBCEC4 cell line.

E. coli 1230 and *C.koseri* 48 were used as negative and positive controls. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments.

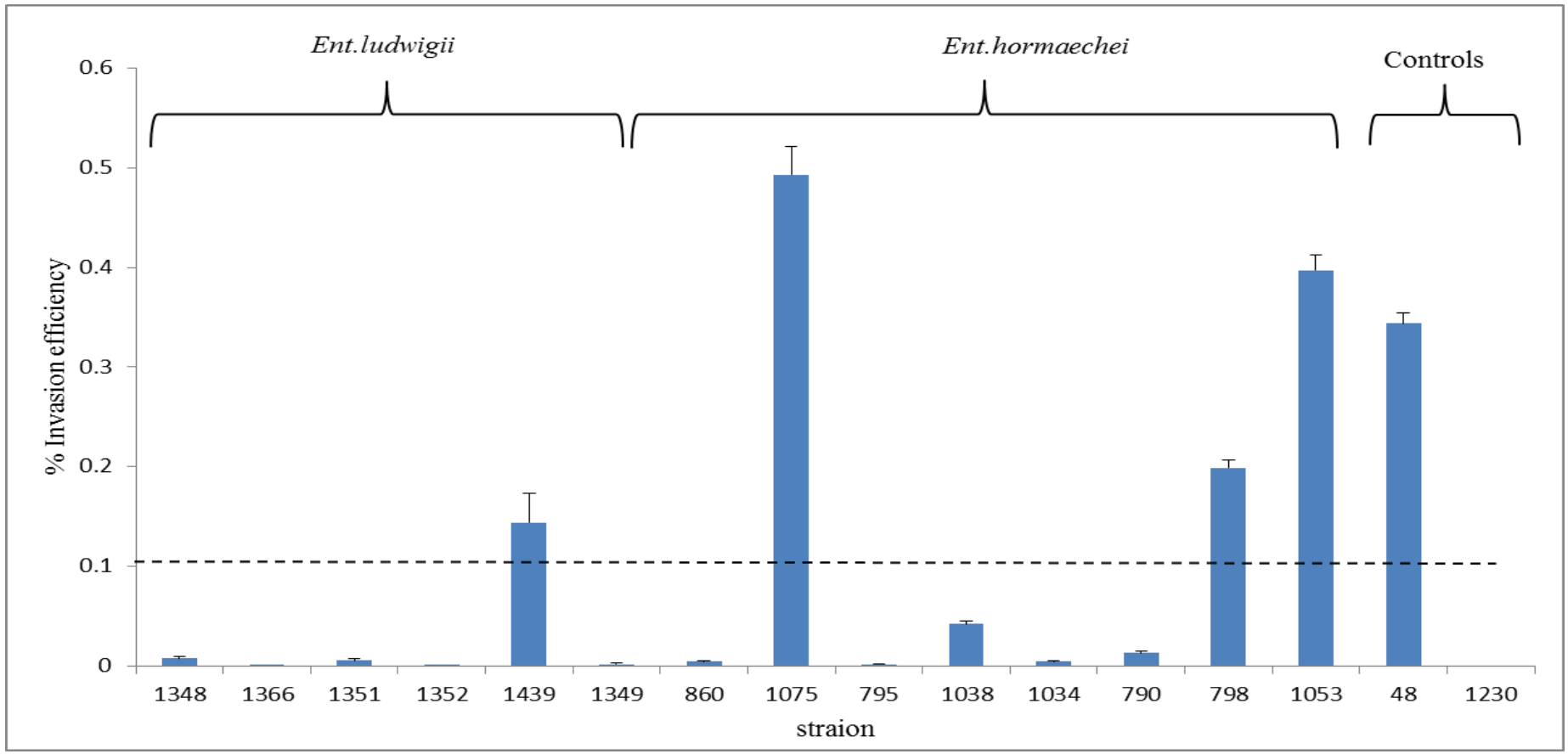


Figure 5.8: Invasion of *Ent. ludwigii* and *Ent. hormaechei* to the mammalian rBCEC4 cell line.

E. coli 1230 and *C. koseri* 48 were used as negative and positive controls. Data represents the mean of results of two independent experiments in triplicate. Error bars represent the standard deviation of two independent experiments.

5.3.7 U937 macrophage uptake and persistence studies

After invasion, a number of bacterial pathogens have the ability to survive and replicate within host cells. The host immune response utilizes phagocytic cell as a critical line of defence against infection. The capability of a pathogen to persist and replicate within macrophages is a potent way of evading the defence mechanisms of the host.

Three strains of *Ent. hormaechei* out of eight strains and 1/2 strain of *Ent. cloacae* persisted in macrophages for 48 hours. In contrast, *Ent. cloacae* strain 779 did not persist after a 24 hours period. *Ent. hormaechei* 798 strain demonstrated a high level of persistence at 24 hours, while *Ent. cloacae* 789 strain showed high replication at 48 hours.

Ent. hormaechei 798 strain displayed a high level of replication. All *Ent. ludwigii* and *K. oxytoca* strains showed no uptake by U937 macrophage cells, or were rapidly killed following uptake (Figure 5.9).

The only significant replicate was observed at 48 minutes for strain 798 ($P < 0.05$).

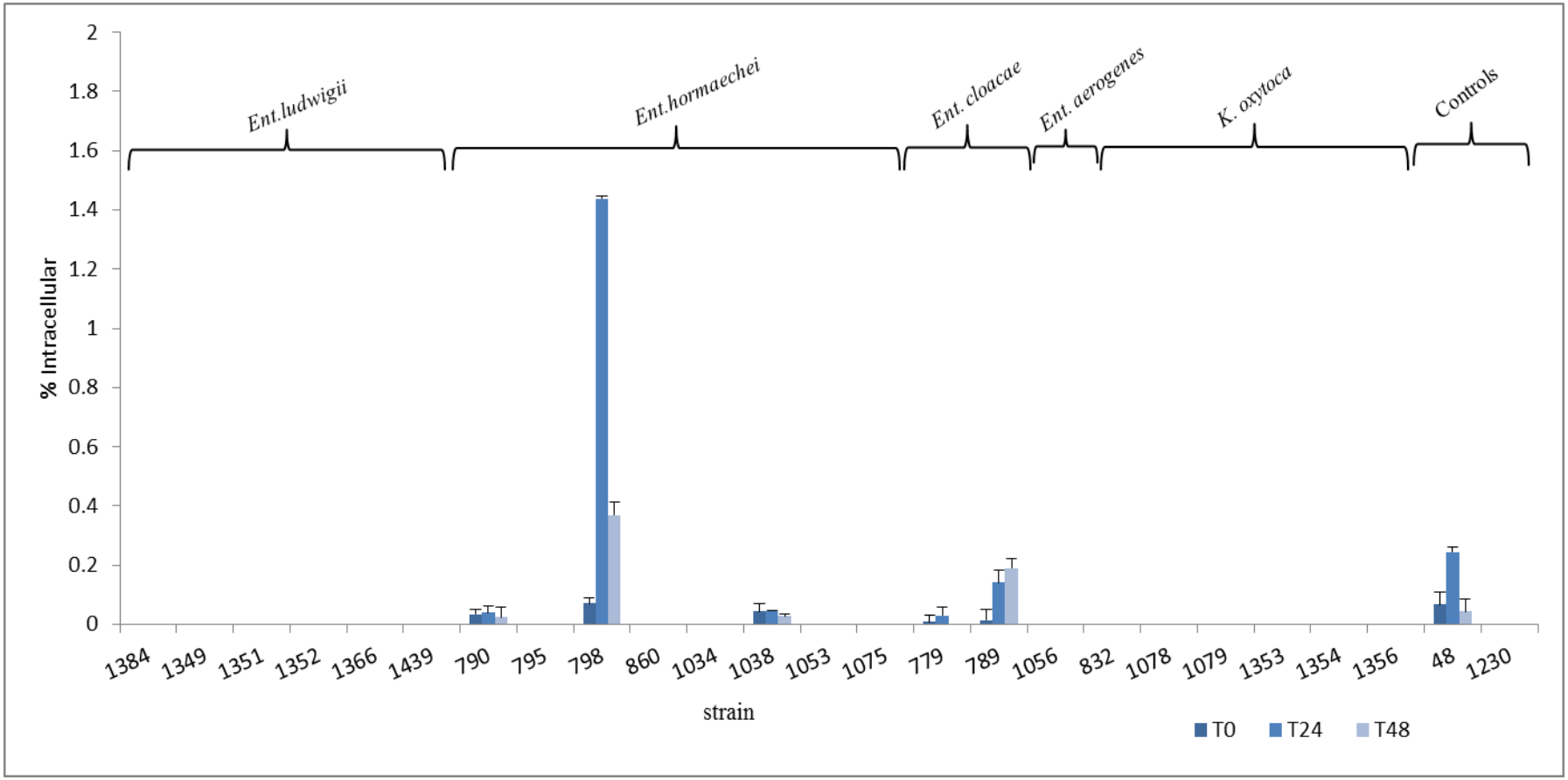


Figure 5.9: *Ent. ludwigii*, *Ent. hormaechei*, *Ent. aerogenes*, *Ent. cloacae* and *K. oxytoca* persistence in U937 macrophage cells after 45 minutes, 24h and 48 h. *E. coli* 1230 and *C. koseri* 48 were used as negative and positive controls. Data are means ± standard errors of two independent experiments performed in triplicate of two independent experiments.

5.3.8 Summary of potential risk for *Enterobacteriaceae*

Evaluation of the ability of opportunistic pathogenic bacteria to attach and invade Caco-2, HBMEC and rBECE4 cells and survive within macrophages are an important consideration. The data for these results was summarised in Table 5.1. The potential risk for 23 strains of *Enterobacteriaceae* was determined and categorised as high, moderate and low with regard to their virulence potential. Six strains showed high potential risk; 3 of the strains persisted within macrophages which included *Ent. hormaechei* strains 798, 1038 and 790, while an other 3 strains, *Ent. hormaechei* 1053 and 1075 and *Ent. ludwigii* 1439, were able to invade rBMCE4 at high levels. *Ent. hormaechei* 798 and 1075 strains also showed high invasion to rBMCE4 and HBMEC respectively. Two strains of *Ent. cloacae* (779 and 789) showed moderate risk. The rest of the strains showed low potential risk.

Table 5.1: Summary of virulence factors for *Ent. hormaechei*, *Ent. ludwigii*, *Ent. cloacae*, *Ent. aerogenes* and *K. oxytoca*.

Species	Strain	Source of isolates	Neonate Number	Period of isolation	PFGE cluster	Neonates of patients isolates represented	Caco-2		Macrophage response	rBMCE4		HBMEC		Potential risk
							attachment	invasion		attachment	invasion	attachment	invasion	
Ent. hormaechei	798	EFT	18	same day	Ebh6	6	Moderate	Low	Persist	Low	High	Moderate	Low	High
"	1053	"	117	1 day	Ebh3	2	Low	High	Killed	High	High	Moderate	Low	High
"	1075	"	117	1 day		2	Low	Low	Killed	High	High	Low	High	High
"	1038	"	113	1week	Ebh7	2	Low	Low	Persist	High	Low	Moderate	Very low	High
"	790	"	4	one month	U	1	Low	Low	Persist	Low	Low	Low	Very low	High
Ent. ludwigii	1439	CSF	A1	"	N/A	1	Low	Very low	Killed	Moderate	High	Low	Very low	High
Ent. cloacae	779	EFT	1	1 week	Ebl1	2	Low	Low	Persist -Killed	N/A	N/A	N/A	N/A	Moderate
"	789	"	12	"		2	Low	Very low	Replicates-Persist	N/A	N/A	N/A	N/A	Moderate
Ent. hormaechei	795	"	17	same day	Ebh6	6	Low	Low	Killed	High	Very low	Moderate	No	Low
"	860	"	52	"	Ebh4	3	Moderate	Very low	Killed	High	Very low	Moderate	Very low	Low
"	1034	"	110	"	Ebh2	2	Moderate	Very low	Killed	Moderate	Very low	Moderate	Very low	Low
Ent. aerogenes	1056	"	118	1 week	Eba1	2	Low	Low	Killed	N/A	N/A	N/A	N/A	Low
Ent. ludwigii	1348	MBM	NK	NK	U	NK	Moderate	Very low	Killed	High	Very low	Moderate	No	Low
"	1349	"	NK	"	"	"	Moderate	No	Killed	Low	Very low	Low	No	Low
"	1351	"	NK	"	"	"	Moderate	Very low	Killed	High	Very low	Moderate	No	Low
"	1352	"	NK	"	"	"	Low	No	Killed	Moderate	No	Low	No	Low
"	1366	"	NK	"	"	"	Moderate	No	Killed	High	No	Moderate	No	Low
K. oxytoca	832	EFT	36	~1 week	Kc5	2	Low	Very low	Killed	N/A	N/A	N/A	N/A	Low
"	1078	"	120	NK	Kc3	NK	Low	Low	Killed	N/A	N/A	N/A	N/A	Low
"	1079	"	121	"		"	High	Very low	Killed	N/A	N/A	N/A	N/A	Low
"	1353	MBM	NK	"	U	"	Moderate	Very low	Killed	N/A	N/A	N/A	N/A	Low
"	1354	"	"	"	"	"	Low	Very low	Killed	N/A	N/A	N/A	N/A	Low
"	1356	"	"	"	"	"	High	Very low	Killed	N/A	N/A	N/A	N/A	Low

EFT: Indicates enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates cerebrospinal fluid. NK: unknown.

5.3.9 Patterns of adherence of *Enterobacteriaceae* to Caco-2 cells

Giemsa staining was used to determine the adhesion patterns of *Ent. ludwigii, Ent. hormaechei, Ent. cloacae, Ent. aerogenes* and *K. oxytoca* to Caco-2 human epithelial cells. Three different adhesion patterns could be distinguished on Caco-2 human epithelial cells:

(i) Aggregative adhesions: Large clusters of adhering bacteria which cover the cell surface, observed for 6/8 strains of *Ent. hormaechei* and 1/6 strains of *K. oxytoca* (see Table 5.2, Figure 5.10 (A)).

(ii) Diffuse adhesions: Bacteria distributed over the cell surface; observed in only one strain ie, *Ent. aerogenes* (see Table 5.2, Figure 5.10 (B)).

(iii) Localized adhesions: Distribution in small groups; observed in all *Ent. ludwigii* and *Ent. cloacae* strains, 5/6 strains of *K. oxytoca*, 2/8 strains of *Ent. hormaechei;* (see Table 5.2, Figure 5.10 (C)).

The strains showed the same adhesion patterns belonging to the same pulsotype (see Table 5.2). Examples of the different patterns of adhesion as observed on Caco-2 human epithelial cells can be seen in Figure 5.10 A, B and C.

Table 5.2: Description patterns of adherence of *Enterobacteriaceae* to Caco-2 cells.

Species	Strain	Source of isolates	PFGE cluster	Adherence pattern
Ent. ludwigii	1348	MHM	U	Localized
"	1349	"		"
"	1351	"		"
"	1352	"		"
"	1366	"		"
"	1439	CSF		"
Ent. hormaechei	790	EFT	U	Aggregative
"	795	"	Ebh6	"
"	798	"		"
"	860	"	Ebh4	"
"	1034	"	Ebh2	"
"	1038	"	Ebh7	Localized
"	1053	"	Ebh3	”
"	1075	"		Aggregative
Ent. cloacae	779	EFT	Ebl1	Localized
"	789	"		"
Ent. aerogenes	1056	EFT	Eba1	Diffuse
K. oxytoca	832	"	Kc5	Localized
"	1078	"	Kc3	"
"	1079	"		"
"	1353	MBM	U	"
"	1354	"		"
"	1356	"		Aggregative

EFT: Indicates enteral feeding tube, MBM: Indicates human mastic breast milk and CSF: indicates cerebrospinal fluid. Ebh2-4, 6 and 7; Ebl1 and Kc3 and 5 indicates PFGE cluster groups, U: Unique.

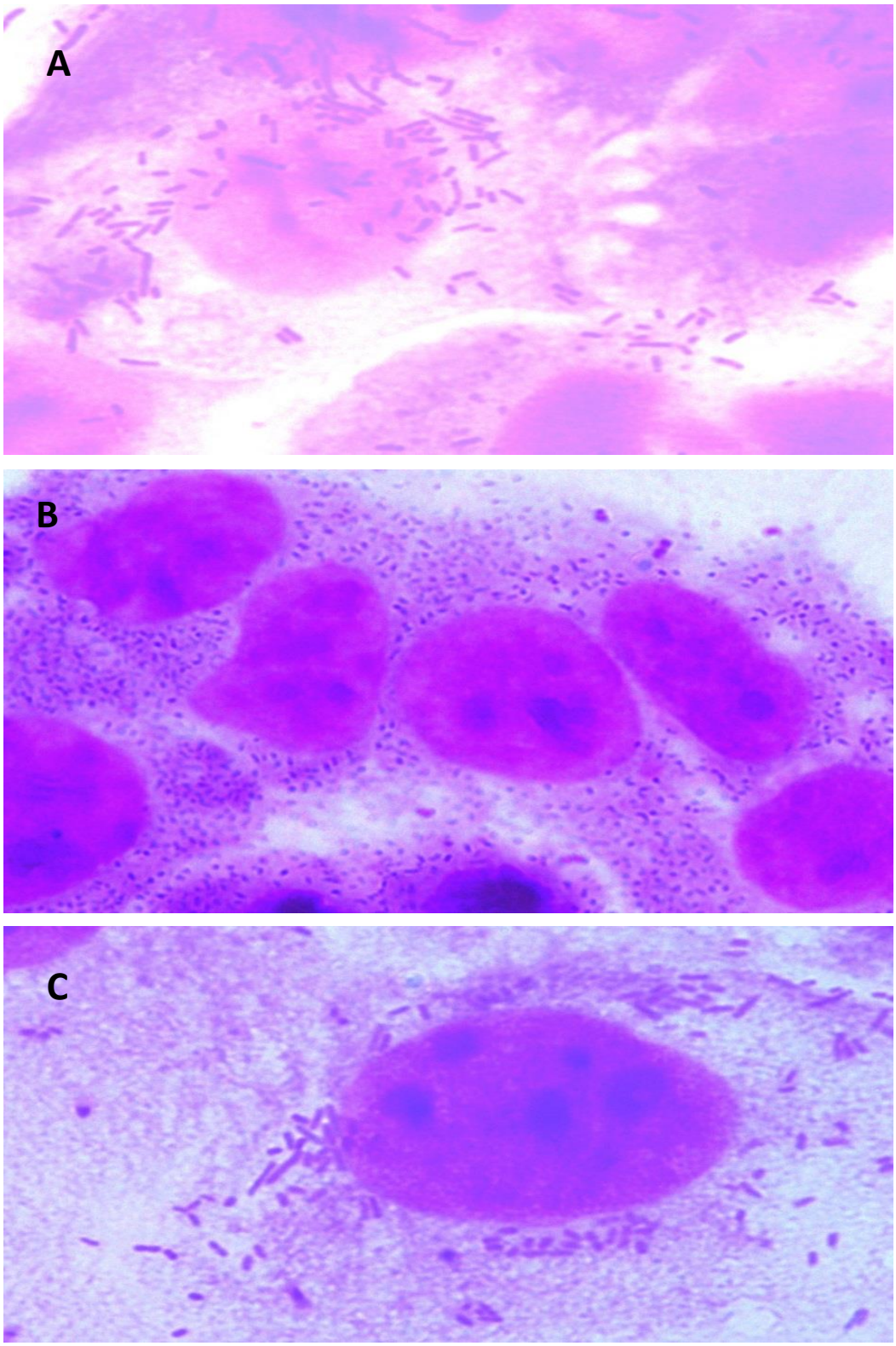


Figure 5.10: Light microscopy of Giemsa stained Caco-2 human epithelial cells, showing the three types of bacteria adherence patterns, after 3 hours incubation of 10^8 bacteria. (A) Aggregative adhesion, (B) Diffuse adhesion, and (C) Localized adhesion. Magnification is x200.

5.3.10 Haemolysin production

Seventeen out of the twenty-three strains showed β- haemolysis on horse blood agar and 21/23 strains showed α- haemolysis on sheep blood agar. Six isolates showed no haemolysis on horse blood agar whereas 1 strain presented the gamma type of haemolysis (non- haemolysis) on sheep blood agar, (Table 5.3).

5.3.11 Serum sensitivity

All *Ent. cloacae, Ent. aerogenes, K. oxytoca* and 4/6 strains of *Ent. ludwigii* and 6/8 strains of *Ent. hormaechei* were resistant to human serum (log differentiation was $\geqslant$ - 0.09) and the high resistance to serum was observed by *Ent. ludwigii* strain 1349, *Ent. cloacae* strain 789 and *K. oxytoca* strains 1354 and 1356 (log differentiation was $\geqslant$1). However, 4 strains were intermediate to human serum which were *Ent. ludwigii* strains 1351 and 1439 and *Ent. hormaechei* strains 1053 and 1075.

Salm. Enteritidis strain 358 and *E. coli* strain 1230 were completely resistant and sensitive to serum, respectively. The results were presented as a viable count (log_{10}cfu/ml) by using log differentiation between the viable count of bacteria at time zero and after 4 hours. The results are shown in Table 5.3.

5.3.12 Siderophore production

Bacteria need iron for a variety of metabolic processes, where the bacteria synthesise and secrete small organic molecules called siderophores which then actively chelate iron. All strains *Ent. ludwigii, Ent. hormaechei, Ent. cloacae, Ent. aerogenes* and *K. oxytoca* were positive for siderophores production, which was represented by orange halos around the colony growth. *Y. enterocolitica* 8081 (positive control) showed a positive result as well. The results are presented in Table 5.3.

5.3.13 Determination of high pathogenicity island

The uptake iron genes *irp1* and *irp2* were absent from all tested strains while gene *fyuA* was detected in all *Ent. cloacae* strains, *Ent. aerogenes* strains, 4/6 strains of *Ent. ludwigii*, 1/8 strains of *Ent. hormaechei*, and 4/6 strains of *K. oxytoca* (Figures 5.11 and 5.12, Table 5.3).

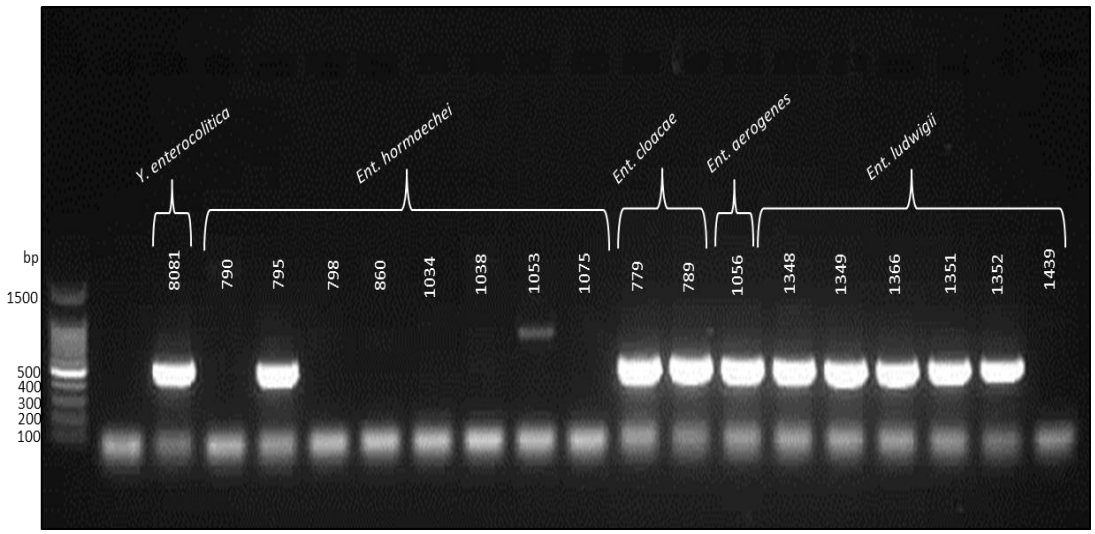


Figure 5.11: lane 1: ladder 100bp; lanes 2 negative control; lane 3: PCR product of *fyuA* gene size 547bp using *Ent. hormaechei*, *Ent. cloacae*, *Ent. aerogenes* and *Ent. ludwigii* DNA. Under the UV light, using the In Genius® gel documentation system (Syngene; UK) DNA samples were visualized. *Y. enterocolitica* strain 8081 was used as positive control.

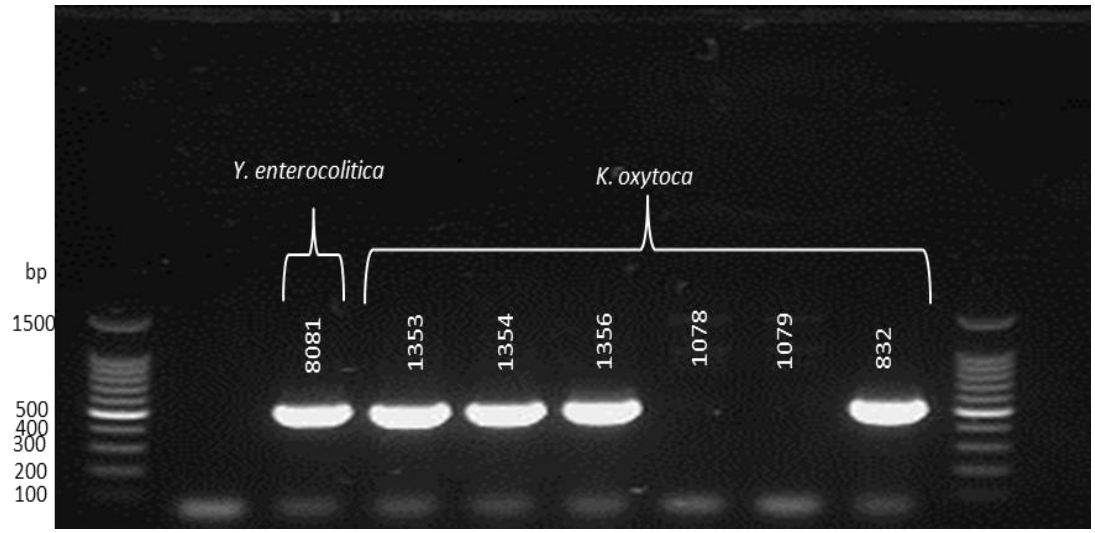


Figure 5.12: lane 1: ladder 100bp; lanes 2 negative control; lane 3: PCR product of *fyuA* gene size 547bp using *K. oxytoca* DNA. Under the UV light, using the InGenius® gel documentation system (Syngene; UK) DNA samples were visualized. *Y. enterocolitica* strain 8081 was used as positive control.

Table 5.3: Description of haemolysin production (horse/sheep blood), serum sensitivity determination and siderophore production.

Species	Strain	Haemolysin production		Serum sensitivity		Siderophore production	Uptake iron genes		
		horse blood	sheep blood	Log differentiation cfu/ml	Result		*irp1*	*irp2*	*fyuA*
Ent. ludwigii	1348	–	α	0.76	R	+	–	–	+
"	1349	–	α	1.05	R	+	–	–	+
"	1351	β	α	– 0.06	I	+	–	–	+
"	1352	β	α	0.67	R	+	–	–	+
"	1366	β	α	0.37	R	+	–	–	–
"	1439	β	α	– 0.05	I	+	–	–	–
Ent. hormaechei	790	β	α	0.90	R	+	–	–	–
"	795	–	α	0.82	R	+	–	–	+
"	798	β	α	0.15	R	+	–	–	–
"	860	β	α	0.10	R	+	–	–	–
"	1034	β	α	0.66	R	+	–	–	–
"	1038	β	α	0.04	R	+	–	–	–
"	1053	β	α	– 0.59	I	+	–	–	–
"	1075	β	α	– 0.30	I	+	–	–	–
Ent. cloacae	779	–	α	0.09	R	+	–	–	+
"	789	–	α	1.16	R	+	–	–	+
Ent . aerogenes	1056	β	α	0.84	R	+	–	–	+
K. oxytoca	832	β	–	0.90	R	+	–	–	+
"	1078	β	α	0.88	R	+	–	–	–
"	1079	β	α	0.86	R	+	–	–	–
"	1353	β	α	0.98	R	+	–	–	+
"	1354	–	α	1.26	R	+	–	–	+
"	1356	β	α	1.00	R	+	–	–	+
Strep. pyogenes	NCTC 9994	β	β	N/A	N/A	N/A	N/A	N/A	N/A
E . coil	1230	N/A	N/A	–2.40	S	N/A	N/A	N/A	N/A
Salm . Enteritidis	358	N/A	N/A	1.23	R	+	N/A	N/A	N/A
Y. enterocolitica	8081	N/A	N/A	N/A	N/A	+	+	+	+

(α) indicated positive/partial haemolysis and greenish color around the colonies. (ß) indicated to clearing around colonies on blood agar. (–) indicated a lack of clearing or another change around colonies on blood agar (non-haemolysis) or indicated absent gene. (+) indicated positive haemolysis. (R) indicated serum-resistant strains; (I) indicated serum-intermediate strains. The serum sensitivity data represents the mean of two independent experiments in duplicate.

5.3.14 Determination of antibiotic susceptibility testing by the disc diffusion method

All *Ent. ludwigii* strains were resistant to ampicillin, doxycycline, cephalothin, cefoperazone and cefotaxime. The exception was strain 1439 which was intermediate to cefuroxime and sensitive to cefoperazone. Most of *Ent. ludwigii* strains showed sensitivity to streptomycin, co-amoxiclav, imipenem, meropenem, ciprofloxacin, chloramphenicol and cefpodoxime. The exceptions were strains 1348 and 1439, which were resistant to streptomycin and intermediate to co-amoxiclav, respectively; and strains 1349 and 1439, which were both resistant to chloramphenicol. *Ent. ludwigii* strains showed intermediate resistance to the rest of the antibiotics tested. A summary of results is given in Table 5.4. All *Ent. ludwigii* strains were not able to produce ESBLs.

All *Ent. hormaechei* strains were sensitive to amikacin and ciprofloxacin. In contrast, all *Ent. hormaechei* strains were resistant to ampicillin, co-amoxiclav, doxycycline, cephalothin, cefuroxime and cefotaxime, excluding 2 strains 790 and 1075 which were resistant to co-amoxiclav (Table 5.4). The combination disc method was used to detect ESBLs production. Three strains of *Ent. hormaechei* (795, 798 and 860) produced ESBLs and they were resistant to 3rd generation cephalosporin. The rest of the strains did not produce ESBLs (see Table 5.4).

All *Ent. cloacae* strains were resistant to gentamicin, streptomycin, doxycycline and cephalothin and intermediate to ampicillin and ceftriaxone. Only 1 strain (ie. 789) was resistant to cefuroxime, cefoperazone and cefotaxime. *Ent. cloacae* strains were sensitive to the rest of the antibiotics tested. The exception was by strains 779 and 789 which were intermediate to ceftazidime and amikacin respectively. *Ent. cloacae* strains did not produce ESBLs (see Table 5.4).

In this study, *Ent. aerogenes* 1056 showed resistance to gentamicin, streptomycin, ampicillin, doxycycline, cephalothin, cefuroxime and cefoperazone, while it was intermediate to ceftriaxone, ceftazidime and trimethoprim. This strain (1056) was sensitive to the rest of the antibiotics tested and was not able to produce ESBLs (see Table 5.4).

All *K. oxytoca* strains were resistant to streptomycin, ampicillin, doxycycline and cephalothin. However, 4/6 strains of *K. oxytoca* were resistant to gentamicin; 5/6 strains which were resistant to cefoperazone; 1/6 strains were resistant to cefotaxime; 5/6 strains were resistant to cefuroxime; and 2/6 strains were resistant to co-amoxiclav, cefuroxime and chloramphenicol. *K. oxytoca* strains were intermediate to amikacin, ceftriaxone, ceftazidime and imipenem, trimethoprim by 1/6, 2/6, 2/6, 4/6 and 4/6 strains of *K. oxytoca* respectively. Most of the *K. oxytoca* showed sensitivity to most of the antibiotics tested (see Table 5.3). All *K. oxytoca* strains were not able to produce ESBLs (Table 5.4).

Across all strains used in this study it was noted that *Ent. ludwigii, Ent. hormaechei, Ent. cloacae, Ent. aerogenes* and *K. oxytoca* showed resistance to doxycycline, cephalothin and cefuroxime and were sensitive to ciprofloxacin and meropenem. Three strains of *Ent. hormaechei* produced ESBLs and *E. coli* NCTC 10418 was sensitive to all antibiotics tested.

Table 5.4: Description of antibiotic resistance profiles of *Enterobacteriaceae* isolates.

Group	Antibiotic	Con.	Interpretation of zone diameters (mm)																					
			Ent. ludwigii						*Ent. hormeachei*								*Ent. cloacae*		*Ent . aerogenes*	*K. oxytoca*					
			1348	1349	1351	1352	1366	1439	790	795	798	860	1034	1038	1053	1075	779	789	1056	832	1078	1079	1353	1354	1356
Aminoglycoside	Gentamicin	10µg	**R**	I	I	**R**	**R**	I	I	I	I	**R**	I	**R**	**R**	I	**R**	**R**	**R**	S	**R**	S	**R**	**R**	**R**
	Amikacin	30µg	**R**	S	I	I	I	S	S	S	S	S	S	S	S	S	S	I	S	I	S	S	S	S	S
	Streptomycin	10µg	**R**	S	S	S	S	S	S	**R**	**R**	S	S	S	S	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
penicillin	Ampicillin	10µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	I	**R**	**R**	**R**	**R**	**R**	**R**	**R**
	Co-amoxiclav	20+10	S	S	S	S	S	I	S	**R**	**R**	**R**	**S**	**R**	**R**	S	S	S	S	S	S	**R**	**R**	S	S
Tetracyclines	Doxycycline	30µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Quinolones	Ciprofloxacin	1µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Cephalosporins 1	Cephalothin	30µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
Cephalosporins 2	Cefuroxime	30µg	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	I	**R**	**R**	**R**	I	**R**	**R**	**R**	**R**
Cephalosporins 3	Ceftriaxone	30µg	I	I	I	I	I	I	S	**R**	**R**	**R**	S	I	I	**R**	I	I	I	I	S	I	**R**	I	I
	Cefoperazone	30µg	**R**	**R**	**R**	**R**	**R**	**S**	S	**R**	**R**	**R**	S	**R**	S	**R**	S	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**
	Cefotaxime	30µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	S	**R**	S	S	S	S	S
	Ceftazidime	30µg	I	I	I	I	I	I	I	**R**	**R**	I	I	I	I	I	I	S	I	S	S	I	I	I	I
	Cefpodoxime	10µg	S	S	S	S	S	S	S	**R**	**R**	**R**	S	S	S	S	S	S	S	S	S	S	S	S	S
Carbapenems	Imipenem	10µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	I
	meropenem	10µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
–	Trimethoprim	2.5µg	I	I	I	I	S	I	S	**R**	**R**	I	I	I	I	I	S	S	I	I	S	I	S	S	S
	Chloramphenicol	30µg	S	**R**	S	S	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	S	S	S	S	S	**R**	**R**	S	S
	ESBL production		no	no	no	no	no	no	no	**yes**	**yes**	**yes**	no	no	no	no	no	no	no	no	no	no	no	no	no

R, resistant; I, intermediate; s, sensitive. (Yes) indicated strain is produce ESBLs and (no) indicated strain is not produce ESBLs.

5.4 DISCUSSION

Pathogenic bacteria use a number of strategies to cause disease in human hosts, some of which include adhesion, colonisation, activation of disease process, serum resistance and toxins production. The most important factor is the ability of bacteria to attach to the host surfaces, such as mucous membranes, gastric and intestinal epithelial or endothelial tissue (Boyle *et al.*, 2003; Finlay and Falkow, 1997; Klemm and Schembri, 2000). *Enterobacteriaceae* are the most common opportunistic pathogens, particularly *E. coli, Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Salmonella*, which are considerably implicated with a wide range of morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012).

In the present study, an attempt was made to determine the relationship between haemolysin production and other virulence associated factors in selected strains of *Enterobacteraceae*. There was a relationship between haemolysin production and other factors, such as serum resistance and siderophore production, which may be associated with neonates' infection.

This study was focused on investigating the potential virulence of *Enterobacteriaceae* from EFT and MBM by determining whether or not they pose a risk of infection to neonates. Forty three *Enterobacteriaceae* composed of *Ent. ludwigii*, *Ent. hormaechei*, *Ent. aerogenes*, *Ent. cloacae*, *Ent. cancerogenus* and *K. oxytoca* strains were chosen to be part of the study as they were the most representative of the species isolated. The potential of virulence for the 43 strains to mammalian cells was determined using attachment and invasion studies of Caco-2 tissue culture cells, while the attachment and invasion rBECE4 and HBMEC tissue culture cells were only studied for 14 strains (*Ent. ludwigii, Ent. hormaechei*). Macrophage survival was studied for 23 strains (*Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca* using the U937 cell line of human monocyte cells.

This study demonstrated that all strains showed the ability to attach to epithelial and endothelial cell lines. The viable counts obtained exhibit the ability of strains to attach to Caco-2, HBMEC and rBECE4 cells. There were differences in the ability of *Ent. ludwigii, Ent. hormaechei, Ent. cancerogenus Ent. aerogenes, Ent. cloacae* and

K. oxytoca to attach to intestinal epithelial cells (Caco-2) (see Figures 5.1-5.4). The viable counts showed that *Ent. cancerogenus* and *K. oxytoca* strains were able to attach to Caco-2 cells at relatively high levels compared with the rest of strains (Figure 5.2). Whereas *Ent. ludwigii* and *Ent. hormaechei* strains attached to rBECE4 cells at higher numbers compared to their ability to attach to HBMEC cells (Figures 5.5, and 5.7). However, all strains were able to attach to the different types of epithelial and endothelial cell lines.

The ability of bacteria to adhere to host surfaces, such as skin, mucous membranes (oral cavity, nasopharynx, urogenital tract), and deeper tissues (lymphoid tissue, gastric and intestinal epithelia, alveolar lining, endothelial tissue), is a key step in the host-pathogen interaction. Hosts produce numerous mechanical forces, such as saliva secretion, coughing, sneezing, mucous flow, peristalsis, and blood flow, which act to wash microbes away from susceptible surfaces. However, microbial pathogens express factors that bind to molecules on various host tissue cells and render the microbe resistant to these mechanical washing forces (Podschun and Ullmann, 1998). As a result, microbial biochemical processes cause diseases, including proliferation, toxin secretion, host cell invasion, and activation of host cell signalling cascades (Wilson *et al*., 2002).

Once adhered to a host surface, some pathogenic bacteria can gain access deep into the host and continue the infection cycle. In this study, there were variations between species in their invasion of Caco-2 cells; for example, all *Ent. hormaechei* were able to invade Caco-2 cells compared to the remaining species. Additionally, there was no direct association between attachment and invasion rates of Caco-2 cells; for example, *Ent. cancerogenus* strains 1350, 1077, 1037, 806 and 1360 and *K. oxytoca* strains 1356 and 1079 had the highest attachment rates, yet 1053 had the highest invasion rates (Figures. 5.1 and 5.3).

Previous studies reported that *K. oxytoca* had been associated with septicaemia, soft tissue, intravenous, meningitis, liver abscess, and gastrointestinal disease. This is commonly observed in immunocompromised hosts or those with underlying conditions (Green *et al.*, 2009). Townsend *et al.* (2008a) demonstrated that virulence traits were associated with *Ent. hormaechei*, and they are capable of invading deep tissues (causing systemic infection and the symptoms that it causes to spread

throughout the systems of the body). Furthermore, *Ent. hormaechei* associated with sepsis were able to invade gut epithelial cells. This demonstrates the virulence potential of *Ent. hormaechei* for this host site and suggests that its ability to invade intestinal cells may be a strong indication of potential virulence and pathogenicity in the neonatal host.

According to Table 5.1, six isolates were classed as high risk, indicating they could increase the exposure of infants to the bacteria from infant formula administered through EFT. These strains were *Ent. hormaechei* strains 790, 798, 1038, 1053 and 1075 and *Ent. ludwigii* strain 1439. In this study, the level of risk was determined according to the ability of strains to invade mammalian cells (Caco-2, HBMEC and rBECE4 cells) and to persist in human macrophages. *Ent. hormaechei* strains 790, 798, 1038, 1053 and 1075 and *Ent. ludwigii* strain 1439 showed low levels of invasion to Caco-2 cells, whereas *Ent. hormaechei* strain 1053 showed a high level of invasion to Caco-2 cells.

On the other hand, the virulence potential of *Ent. hormaechei* and *Ent. ludwigii* strains to invade brain capillary endothelial cells was determined using HBMEC and rBECE4 cells. Interestingly, three strains of *Ent. hormaechei* (789, 1053 and 1075) showed similar invasion to both cell lines (HBMEC and rBECE4); however, *Ent. ludwigii* strain 1439 was able to invade rBECE4 cell line at much higher levels than HBMEC cell line. *Ent. ludwigii* strain 1439 was isolated from a case of neonatal meningitis (CSF) that was obtained from Nottingham QMC hospital. This study is an *in vitro* appraisal evaluating the virulence of the isolate in relationship to clinical symptoms. There is a strong likelihood that the baby could have acquired the strain from the environment, through the skull, as its skull was opened for the brain surgery.

It was possible to directly associate clinical symptoms and results with *in vitro* studies. For *Ent. hormaechei* strains isolated from EFT, there was no clinical data for the patients; however, the ability of *Ent. hormaechei* strains to invade HBMEC and rBECE4 cell lines may be a strong indication of potential virulence and pathogenicity in the neonatal host to cause meningitis. Hervas *et al.* (2001) reported that *Ent. hormaechei* may be associated with meningitis because sepsis and meningitis were associated with *Enterobacter* spp. In addition, this result is consistent with a previous study by Townsend *et al*. (2008b), who studied virulence assays of clinical

isolates with associated patient details and clinical symptoms. They found that *C. sakazakii* strains were able to invade HBMEC and rBCEC4 cells.

Also demonstrated was the ability of selected strains of *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca* to persist in human macrophages. Three strains of *Ent. hormaechei* (790, 1038 and 798) and 2 strains of *Ent. cloacae* (779 and 789) were able to persist and replicate in macrophages for 48 hours. However, *Ent. cloacae* strain 779 showed decreased levels of persistence after 24 hours while *Ent. hormaechei* strain 798 persisted at high levels within the macrophages up to 48 hours and then was killed (Figure 5.9). *Ent. ludwigii, Ent. aerogenes* and *K. oxytoca* strains were killed by macrophages.

These results compared the ability of selected strains to invade mammalian cells. The important finding was that three *Ent. hormaechei* strains (790, 798 and 1038) were able to invade three types of cell lines (Caco-2, HBMEC and rBECE4 cells) (Table 5.1, Figures. 5.3, 5.6, 5.8 and 5.9). These results indicate there could be a relationship between the invasion of epithelial and brain endothelial cells, and also persistence and replication in human macrophage cells. The ability of strains to invade capillary endothelial cells may give access to the brain by crossing the blood-brain barrier which may led to infected neonates. Moreover, the persistance and replication of the strains in human macrophage cells could be important to facilitate host immune evasion and dissemination.

These results were consistent with the study conducted by Townsend *et al*. (2008b), who studied a NICU outbreak of *C. sakazakii* infections in France. They found that all *C. sakazakii* strains were able to attach and invade Caco-2 human epithelial cells, and invade rat brain capillary endothelial cells, and also most of the strains persisted in macrophage cells for 48 hours. They suggested that these features act to facilitate host immune evasion and spreading.

Patterns of adherence of *Enterobacteriaceae* to Caco-2 cells were studied. Selected strains of *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca* did adhere to Caco-2 cells, and there were different patterns of adherence. Whether the pattern of adherence relates to the pathogenicity of the strains is unknown. In this study, there was no relationship between types of adherence

patterns and ability to invade the Caco-2 human epithelial cells (as shown in Table 5.2, Figure 5.10 A, B and C).

A number of virulence factors, that including haemolysins production, serum sensitivity determination, siderophore production and antimicrobial susceptibility, were investigated.

Haemolysin production is one of the toxins that are secreted by pathogenic bacteria and may play an essential role in disease. Haemolysins are classified to three different types of haemolysins: α-haemolysin, β-haemolysin and γ-haemolysin. These are found in most clinical isolates, such as *E. coli* and *Serratia* species (König *et al*., 1987; Welch, 1987; Schmidt *et al*., 1995). The present study shows that all *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca* strains were α-haemolytic on sheep blood agar, except *K. oxytoca* strain 832, while β-haemolysin was observed on horse blood by 17/23 strains. Previous studies have shown that the most important virulence factor commonly expressed by extra-intestinal pathogenic bacteria, such as *E. coli,* is α-haemolysin (Balsalobre *et al*., 2006).

Another study by Ring *et al*. (2002) demonstrated that β-hemolysin plays an important role for the result of group B streptococcal sepsis by contributing to liver failure and high mortality. Thus, these results suggest that the strains have the ability to cause haemolysis and thereby damage the cell membrane of RBCs through production of β-haemolysis which is a potential risk factor to neonatal health.

The sensitivity to normal human serum of isolated *Enterobacteriaceae* was determined. All *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae,* and *K. oxytoca* strains were serum resistant, except four strains, *Ent. ludwigii* strains 1351 and 1439 and *Ent. hormaechei* strains 1053 and 1075, which survived in blood serum at an intermediate rate. A common feature of many different species causing bacteremia is the capability to avoid the bactericidal effects of human serum (Porto *et al*., 1989; Hol *et al*., 1995; Hoe *et al*., 1999).

Several previous studies found that a common feature of meningococci isolated from blood or cerebrospinal fluid is serum resistance (Figueroa and Densen, 1991). Similarly, the clinical isolated of *V. vulnificus* exhibited the ability to survive in human serum (Bogard and Oliver, 2007). Furthermore, *Ent. hormaechei* nosocomial

outbreak strains isolated from different neonates in a California hospital showed serum-resistance (Townsend *et al*., 2008b). The finding in the current study could be explained by the ability of strains to survive in blood serum which possibly indicates an increased risk to neonates.

Iron is an important element for pathogenic bacteria to grow, replicate and progress an infection (Braun and Winkelman, 1987). Bacterial ability to obtain iron from the host is one of the determinants for the virulence of a pathogen. All *Enterobacteriaceae* isolates showed active siderophores production. The identification and characterization of the molecular virulence factors of genes that allow pathogens to cause infection in the hospital pathogenic bacteria have been used (Wilson *et al.*, 2002). Since all of the investigated *Enterobacteriaceae* produce siderophores, the *irp1, irp2, fyuA* genes in HPI were detected as uptake iron genes. The siderophore yersiniabactin production is connected with virulence in *Yersinia* species (Schubert *et al.*, 2000).

The uptake iron gene *fyuA* was detected in all *Ent. cloacae* strains, and *Ent. aerogenes* strains, 4/6 strains of *Ent. ludwigii*, 1/8 strains of *Ent. hormaechei*, and 4/6 strains of *K. oxytoca*, while genes *irp1* and *irp2* were absent from all tested strains (Figures 5.11 and 5.12, Table 5.2). The interesting finding is that the strains of *Ent. ludwigii*, *Ent. hormaechei*, and *K. oxytoca* produced siderophores phenotypically, while genetically lost genes *irp1*, *irp2* and *fyuA*. This finding may suggest that these strains possibly use different iron uptake systems.

All *Ent. ludwigii*, *Ent. hormaechei*, *Ent. aerogenes*, *Ent. cloacae*, and *K. oxytoca* strains showed multiple resistances to a broad spectrum of antibiotics. The interesting finding was three strains of *Ent. hormaechei* (795, 798 and 860) exhibited resistant to aminoglycoside, pencillin, tetracycline, chloramphenicol, quinolones and cephalosporins 1, 2 and 3, but were sensitive to carbapenems. In addition, the high levels of cephalosporins resistance in *Enterobacteriaceae* may be because of hyper production of chromosomal AMPC. This finding is consistent with Hurrell *et al.* (2009b) who reported that many of *Ent. hormaechei* strains isolated from ENT from QMC and NCH hospitals were resistant to the 3rd generation cephalosporins, ceftazidime and cefotaxime, and ESBLs were detected. This demonstrates that neonates are directly exposed to antibiotic resistant strains in NICUs during feeding.

Chapter 6: GENERAL DISCUSSION

Recently, the incidence of neonatal infections caused by *Enterobacteriaceae* has been increasing and they are now considered as major causative agents in NICU outbreaks (Gastmeier *et al.,* 2007; McGuire *et al.,* 2004; Kaufman *et al.,* 2004). *E. coli, Enterobacter* spp., *Klebsiella* spp., *Serratia* spp. and *Salmonella* are considered as the most common opportunistic pathogens which are implicated with morbidity and mortality (Friedland *et al*., 2003; Adamson *et al*., 2012). These organisms are able to cause many different types of infections such as sepsis, brain meningitis, pneumonia and urinary tract infections, particularly in intensive care units (ICU) (Kollef *et al*., 1999; Ibrahim *et al*., 2000). *Enterobacteriaceae* such as *E. coli, Klebsiella, Proteus, Serratia* and *Citrobacter* spp. have the ability to cause nosocomial infections. Several of these species are the members of human normal flora such as *E. coli* and *Ent. cloacae* and possibly can cause infections via selection following an empiric antimicrobial regime (Iversen and Forsythe, 2004a; Stoll *et al*., 2005).

This project concerns the risk assessment to neonates from ingested *Enterobacteriaceae* from MBM and EFT. This study was set up with the aim of assessing and characterising strains from these two sources and also, to study physiological traits associated with exposure and to determine virulence traits. The strains from the EFT of neonates in intensive care units from the UK have been published by NTU (Hurrell *et al*., 2009b), whereas the strains from MBM were from an external source from Spain (Delgado *et al.,* 2008). Strains isolated from MBM and EFT included bacteria which are potentially pathogenic to neonates. The present study is a continuation of these studies and has further investigated and characterised these strains. Later on, an additional member of *Enterobacteriaceae, K. pneumoniae* isolated from EFT (obtained from Jordan) was also included in this study. These *K. pneumoniae* strains were analysed, but only for PFGE profiling due to time constraints. *Enterobacteriaceae* from EFT and MBM in this study were initially identified using phenotyping methods and compared with genotyping.

The PFGE technique was used to determine if isolates are indistinguishable or closely related. This technique is based on multilocus enzyme restriction; as described by Tenover *et al.* (1995). There are many laboratories which use PFGE to determine strain relatedness and to identify the source of a strain or outbreak (Agasan *et al.,* 2002; Bender *et al*., 2001).

A total of thirty-four pulsotypes of *Enterobacteriaceae* from EFT (21 from the UK and 10 from Jordan) and 3 MBM pulsotypes were identified from neonatal enteral feeding tubes and mastic human breast milk using PFGE. The same pulsotypes were disseminated across enteral feeding tubes of different infants in the same NICUs. Furthermore, the same pulsotypes were spread between enteral feeding tubes of infants in the same NICUs, indicating the same origins, such as: environment, infant formula or carers. The exception was one cluster of Jordanian strains which was the Kp2 pulsotype. This pulsotype showed an unexpected result because the strains were isolated from two different hospitals (the KAH and PRH Jordan). According in Figure 1.1 in section 1.6 the explanation for this observation could be that the same brand of infant formula "bebelac" was used in the two hospitals in Jordan which could have been contaminated with bacterial strains in the Kp2 cluster. The KAH and PRH hospitals fed the babies with bebelac in May and July respectively and this clone may have cross contaminated the neonatal enteral feeding tubes for a long time period (from May to December) and infected an enormous number of neonates. In addition, health care worker (e,g. Doctors, Nurses) may be working in tow hospital and may transmission of strains between two hospital. Another source could be medical equipment supplier that used medical equipment, healthcare equipment, hospital equipment, and more. These two hospitals may be provided with equipment from same company. However, this assumption needs verification by using a different restriction enzyme in the PFGE.

These findings are consistent with previous studies which stated that PIF is not a sterile product and can possibly be contaminated with pathogens that can lead to serious disease in infants who are less than one year old. The potential for contamination of PIF during production has been studied by FAO/WHO (2004 and 2006) and several surveys of the PIF drew attention towards the pathogens that may contaminate this infant foodstuff (Iversen and Forsythe, 2004; Muytjens *et al.*, 1988). In 2004, the report by the Food and Agriculture Organization of the United Nations and the World Health Organization concluded that *Sal.* Enterica and *C. sakazakii* are the microorganisms of greatest concern in PIF (FAO/WHO, 2004 and 2006).

Another previous study by Morgan *et al*. (2012) is agreement with this study they reported that the contamination of healthcare workers' protective clothing during routine care of patients with multidrug-resistant organisms is most frequent with *A.*

baumannii. The major determinant of transmission to healthcare workers' gloves or gowns was environmental contamination.

The EFT strains (UK) were isolated over the period from January to November 2007. This study demonstrated that twenty-one pulsotypes of *Enterobacteriaceae* were isolated from contaminated neonatal enteral feeding tubes for which no clear sources were known. These pulsotypes included; seven *Ent. hormaechei,* six *Ent. cancerogenus,* six *K. oxytoca,* one *Ent. cloacae* and one *Ent. aerogenes* pulsotype. Previous studies stated that some of these most commonly faced species; *Ent. aerogenes*, *Ent. cloacae*, *Ent. agglomerans* and *C. sakazakii* have been associated with infections in humans (Andresen *et al*., 1994; Burchard *et al*., 1986; Chow *et al*., 1991; Gallagher, 1990; Gaston 1988; Haddy *et al*., 1991; Hawkins *et al*., 1991; Stenhouse *et al*., 1992).

This study has shown that three different clusters of *K. oxytoca, Ent. cancerogenus* and *Ent. hormaechei* strains were isolated over a wide time period and comprised of a large number of strains. This indicates that a large number of strains were colonising the enteral feeding tubes; for instance, pulsotypes group Kc1 and Kc6 of *K. oxytoca* comprised 6 and 13 strains, isolated over a one month period and a two weeks period and isolated from two and five patients, respectively [Appendix 5]. Similarly, for two large pulsotype groups Ebc2 and Ebc3 of *Ent. cancerogenus*, each group included 13 and 21 strains and was isolated over a prolonged period of three months and two months, respectively [Appendix 4]. In addition, pulsotype group Ebh5 of *Ent. hormaechei* comprised 8 strains, isolated during a one month period from 16 October to 19 November from three patients.

These findings may indicate the cross-transmission of indistinguishable clones of each pulsotype among different patients for long periods in the same NICUs. However, the rest of the pulsotypes for the *Ent. hormaechei*, *Ent. cancerogenus* and *Klebsiella* spp. showed a large variety of PFGE profiles (Figures 3.1-3 and Figure 3.6). This indicates the enteral feeding tubes were colonised by a broad range of *Enterobacteriaceae*. The diversity in PFGE profiles indicates that the EFT could be contaminated from different sources. The significant explanation for this finding is that the same NICU was contaminated by the same pulsotypes which spread between the enteral feeding tubes of infants and colonised different patients. Thus, the

contamination of feeding tubes could be from the same sources, such as: environment, infant formula or carers, (Figure 1.1 in section 1.6).

These findings are in agreement with Muytjens *et al*. (1988) who isolated *Enterobacteriaceae* from PIF including *Ent. hormaechei, Ent. cloacae, E. coli, K. pneumoniae* and *K. oxytoca*. Likewise, studies by Caubilla-Barron *et al.* (2007) and Iversen *et al*. (2008) recovered *C. sakazakii* and *Salmonella* from contaminated PIF and linked them with neonatal infections.

The increase in bacterial growth and cross contamination in NICUs is likely to be affected by the reconstitution temperature of PIF for infants. Forsythe, (2009) reported that in NICUs, PIF (fortified breast milk and reconstituted powdered infant formula) was prepared at room temperature, not at the higher temperatures required when the formula was needed. The procedure used within NICUs may be different across the UK.

Another potential source of bacterial cross transmission between the patients is the contaminated health care worker. The most common organisms which can be transmitted from patient to patient on the hands of the health care team are the *Enterobacteriaceae* (Brady, 2005). Health care workers were considered as the source of a nosocomial outbreak of colonisation and infection with a strain of *Se. marcescens* (de Vries *et al.,* 2006). Paper can be source for cross-contamination of bacterial pathogens in medical settings if current recommendations on hand hygiene aren't meticulously followed (H"ubner *et al.*, 2011).

Outbreaks of MRSA colonisations and infections in NICUs have previously been epidemiologically linked with health care workers infected with chronic otitis external (Bertin *et al*., 2006). Another study indicated that Gram-negative bacilli which were previously linked with infection in neonates could be observed on the nurses's hands even after normal hand hygienic practices were followed (Waters *et al*., 2004). Likewise, the immediate environment of the patient, such as patient gowns, bed linen, bedside furniture, and other objects, become contaminated with patient flora (Bonten *et al*., 1996; Samore *et al*,. 1996).

In this study, a collection of twenty *Enterobacteriaceae* strains isolated from MBM were also characterised by the PFGE technique. Six strains of *Ent. cancerogenus*

were divided into three pulsotypes; Enc1- Enc3, and the rest of the strains were unique. The interesting finding is each pulsotype (Enc1- Enc3) consisted of two strains and were found to have 78%, 89% and 92% similarity patterns, respectively (Figure 3.5). The PFGE result showed the strains differ by only 3 bands and they are probably two isolates of the same clone for each pulsotype. Additionally, these six strains were also found to be nearly identical by using 16S rDNA sequence analysis. The results of the MBM isolates indicated clonality of six strains belonging to three pulsotypes which are likely to be isolated from the same mother. On the another hand, the explanation for this observation could be that the same clone may have cross contaminated human milk from nurse or breast pump, see Figure 1.1 in section 1.6.

Strains used in this study were identified using both phenotypic (API20E and ID32E) and genotypic (16S rDNA sequence analysis) techniques. Using phenotypic techniques API 20E and ID32E, the strains isolated from EFT, were clustered as Ebl1 and Ebh7 respectively (Table 3.7, part 1 and 2). Each cluster had two strains; *Ent. cloacae* and *K. pneumoniae* belonging to pulsotype Ebl1 and *Ent. amnigenus* and *Ent. cloacae* were clustered under Ebh7. Interestingly, using 16S rDNA sequence analysis, the strains were re-identified as *Ent. cloacae* and *Ent. hormachei*, respectively.

Similarly, four strains misidentified using the phenotypic method ID32E, one of the strains was identified as *E. coli* and three other strains as *Ent. aerogenes* (Table 3.8). However, using PFGE it was shown that all the four strains belonged to Kc1 and Kc6 pulsotype, respectively. Furthur analysis using 16S rDNA sequence analysis, all of these four were re-identified as *K. pneumoniae*.

The MBM strains were also characterised using standard phenotyping methods and compared with genotyping techniques (Table 3.8). The interesting finding was that six strains of *Ent. cancerogenus* were clustered into three pulsotypes (Ecn1- Ecn3) and had previously been misidentified as *K. oxytoca* using a more common biochemical identification kit, ID32E.

Overall, these findings confirm that 16S rDNA sequence analysis is a more reliable identification method in comparison to phenotypic techniques. This is in agreement with previous studies which reported that API 20E and ID32E tests are imperfect

techniques for the identification of bacteria because they could give false-negative and false-positive results (Iversen *et al*., 2004a and b). As in this study, strain misidentification was reported by Iversen *et al*. (2004a and 2004b) who demonstrated that some strains identified as *Ent. sakazakii* using biochemical test actually belonged to diverse species, including two species *Ent. amnigenus* and *Ent. cloacae*.

A selection of species from PFGE analysis were chosen to determine the physiological attributes with respect to stress survival. These species included *Ent. ludwigii*, *Ent. hormaechei*, *Ent. cancerogenus*, *Ent. cloacae*, *Ent. aerogenes* and *K. oxytoca*. The effect of formula type and temperature at 55°C on stress survival of bacteria was determined.

Biofilm formation on plastic surfaces was determined using casein, whey and soy-based infant formulas at 20°C and 37°C, capsule production at 20°C and 37°C and acid tolerance of organisms to pH 3.5 (HCl acidified formula).

The ability of strains to survive at 55°C were determined and most strains survived at 55°C (Table 4.1 and 4.2). An interesting finding was that strains showing the same death rate at 55°C also belonged to the same pulsotype. For example, pulsotypes of Ebc1, Ebc2, Enc3, Ebh6 and Kc3 for *Ent. cancerogenus* strains 845, 848, 1355, 1358, 1363 and 1364, *Ent. hormaechei* strains 795 and 798, and *K. oxytoca* strains 1078 and 1079, each pulsotype comprising two strains respectively, were sensitive to heat tolerance at 55ºC on whey and casein-based infant formula.

Based upon this finding, it may be concluded that these strains have the same source of origin and various feeding tubes were possibly contaminated by the same clone. As the MBM *Ent. cancerogenus* strains (1355, 1358, 1363 and 1364) were isolated from the same mother too, or breast pump, there is also a possibility of ingestion of these strains by neonates where they could have been exposed to the contaminated milk or the infant formula.

This finding is consistent with previous studies that have demonstrated fortified breast milk and reconstituted PIF were prepared in a neonatal care unit at room temperature when required, and not at higher temperatures (Forsythe, 2009). Another study by Rosset *et al.* (2007) reported that, in a study of French hospitals, similar procedures were used by reconstituting the samples with room temperature water.

Besides, the survival of strains at 55ºC possibly is one of the significant traits resulting in infections of neonates in NICUs which was previously defined by Iversen and Forsythe, (2004a) who demonstrated that *C. sakazakii* strains can grow in PIF between 6° and 45°C and the optimisation temperature of growth being 37°C to 43°C. According to a FAO and WHO meeting (2006), when lukewarm water is used for the reconstitution of PIF (if not used immediately) or when the bottles were stored at room temperature and prolonged feeding periods, the relative risk was found to be enhanced.

The most important goals of this study were to determine the ability of bacteria to form a biofilm on the surface of feeding tubes at different temperatures (20°C and 37°C), as well as to investigate whether the biofilm formation is affected by different types of formula, more details in section 1.10, Figure 1.2. The temperature and type of formula may be a mean for the persistent survival of the organism in the neonatal intensive care unit and the factor for possible attachment of the organism to the surface of the enteral feeding tubes. In the current study, most strains in all types of formula formed a high level of biofilm at 37ºC compared with 20ºC, (Figures 4.9, 4.10 and 4.11).

All the three kinds of formula were subjected to biofilm quantification, where all the strains resulted in high biofilm formation in casein-based infant formula when compared with whey or soya-based infant formula. This shows the biofilm formation was affected by the type of nutrition present in the milk and these findings corroborate the previous studies (Kim *et al.,* 2006; Meadows, 1971)

A previous study has reported that the nutrient sources and other media components affect the attachment of the biofilm forming bacteria on the surface of various materials (Hood and Zottola, 1997). Meadows (1971) has shown that casein enhanced the attachment of various organisms (*E. coli, Pseudomonas fluorescens* and *Aeromonas liquefaciens*) to glass surfaces and the same result was also observed for stainless steel surfaces when whey was present (Speers *et al.,* 1985).

On the other hand, the strains in the same pulsotype formed the same quantity of biofilm in whey and casein-based infant formula at 37ºC and 20ºC. For example, *Ent. hormaechei* strains 1053 and 1075 and *Ent. cancerogenus* strains 1355, 1358, 1359, 1360, 1363 and 1364 belonged to corresponding to pulsotypes respectively *viz.*

Ebh3, Enc2, Enc1 and Enc3 (a pulsotype has two strains). The strains formed equivalent biofilm formation at 37ºC and 20ºC in both the whey and casein-based infant formula. *Ent. hormaechei* strains 1053 and 1075 only exhibited the same level of biofilm in whey-based infant formula at 37ºC; Table 4.3. Based upon these findings, it can be concluded that the same strain contaminated different feeding tubes and the same clone has been recovered from the same mother (MBM strains).

The findings of this investigation are in agreement with the study previously conducted by Hurrell *et al*. (2009b) where bacteria were shown to have the potential to break away from the biofilm in clumps and plausibly be a souce of contamination. Therefore when fresh feed is added into the tube lumen, bacterial multiplication and contamination could further be enhanced. When injested by the neonate, the bacteria may be able to survive in the stomach and endure passage to the intestines. The ability of the bacteria to pass through the intestinal cells may be an implication of either the bacteria's virulence or poor immunity in the neonatal host.

Several characteristics of bacteria may add to their capacity to form biofilms and make them resistant to environmental pressures. the ability of bacterial to form biofilm and attachment to mammalian cells based on EPS which is component of colonic acid and cellulose. Similarity, the genes *pgaC*, *yhjN* and *wcaD* have associated with the ability of *E. coli* O157:H7 to form biofilms on plastic surfaces as well as alfalfa sprouts and Caco-2 cell lines, as their deletion resulted in significant reduction in biofilm formation (Matthysse et al. 2008). Moreover, EPS expression in the wells (neonates EFT) could be linked with the capability of bacteria to form biofilm.

Production of capsular material was determined for *Ent. ludwigii*, *Ent. cloacae, Ent. aerogenes, Ent. hormaechei*, *Ent. cancerogenus* and *K. oxytoca* strains by colony appearance on milk agar (whey, casein and soya) at 20°C (after 24 hours and 48 hours) and 37°C (after 24 hours). Most strains produce capsular material on the three types of milk agar (whey, casein and soya) at 37ºC compared with 20ºC. These results indicate that production of capsules around the bacteria depends on the body temperature.

Interestingly, all the species under investigation were able to synthesize capsular material in soya-based infant formula at 37ºC (Table 4.3 and 4.4, Figuer 4.8) rather

than in whey-based or casein-based infant formula. This finding also demonstrates that the composition of the infant formula also influences the synthesis of the capsular material by the bacteria under investigation. For instance, whey-based or casein-based formula were comprised of lactose and soya-based formula contained glucose (section 1.7, Table 1.2). Lactose is a disaccharide derived from the condensation of galactose and glucose, the latter might be needed for the synthesis of the capsular material. Glucose could only be obtained from whey-based or casein-based formula upon breaking down of the oxygen bond, a process which may have slowed down capsular formation in comparison to soya based formula.

This study also revealed the correlation between the formation of the biofilm and the synthesis of the capsular material by all the species of bacteria except *Ent. hormaechei* on casein milk agar at both the temperatures (20ºC and 37ºC). This may be due to the nutrient required for the synthesis of the material which results in the attachment survival and growth of the bacteria on the surface of the feeding tubes. This is in agreement with the previous studies by Van Acker *et al.* (2001) where they reported that infant formula milk may contain a factor which aids the bacteria in attaching to the surface of the stainless steel or feeding tubes for their growth and survival.

Moreover, it is also in agreement with the report by Scheepe-Leberkühne and Wagner (1986), who suggested that the attachment and adherence by bacteria may be due to the synthesis of exopolysaccharides. One other earlier study has also suggested that the copious synthesis of the capsular material contributes to the formation of biofilm in the clinical isolates of *Cronobacter* (Caubilla-Barron *et al*., 2007). Hence the biofilm produced by the bacteria may protect it from desiccation and also aid in masking the bacteria from the host's immune response, resulting in the development of the disease.

There was a correlation between capsular material production by strains on milk agar and uptake and persistence of strains within macrophages. For example, *Ent. cloacae* 779 and 789 produced capsules on three types of infant formula and those strains persisted in microphages. Previous study by Guerry and Szymanski, (2008) demonstrated that capsular material productions may play a role in resistance to

desiccation, serum activity and contribute to the survival of pathogenic bacteria after ingestion by phagocyte cells.

As previously mentioned, the bifilm formation on EFT may ultimately lead to colonisation of neonate's stomach as they the bacteria may get detached as cluster thereby contaminating the feed.

As previously discussed, the organisms originating from the bacterial biofilm on EFT surface detach and break off in clumps. These biofilms and clumps will contaminate any fresh feed in the tube lumen leading to further bacterial multiplication. Bacteria enter the neonate stomach, and clumps of cells may survive passage through the neonate's stomach. Although the acidity in the stomach of an adult is normally high, and kills the majority of ingested bacteria, for the neonates this is not true. The gastric pH of neonate was 3.5 to 4.3 for the residual feeding regimes (Hurrell et al., 2009b).

In this study, all strains were able to survive on two types of infant formula (whey and casein) after adjusting to pH 3.5 for two hours at 37ºC, (Figures 4.12-4.19).

Gastric juice is reported to play a pivotal role in controlling the food-borne infections and this research also backs up this point. The competence of bacteria in enduring the acidic conditions of the stomach implies that the bacteria might be pathogenic, which may result in disease. Earlier studies have shown that the survival rates of a few common food-borne infections were increased when the gastric acidity fell (Peterson, 1989) accompanied by the decrease in the infective dose (Cash *et al.*, 1974; Schlech *et al.*, 1993).

The next part of study determined the virulence potential of strains isolated from MBM and EFT using tissue culture techniques and identified the presence of virulence factors. Mammalian cell lines Caco-2 (colon) are used as representatives of the human intestinal tract whereas HBMEC and rBCEC4 are used as representatives of the human brain. Forty three *Enterobacteriaceae* including *Ent. ludwigii*, *Ent. hormaechei, Ent. aerogenes, Ent. cloacae, Ent. cancerogenus* and *K. oxytoca* strains were chosen to determine their attachment and invasion of Caco-2 tissue culture cells. Fourteen organisms were checked for the ability to attach and invade through the surface of rBECE4 and HBMEC cells. Moreover, the ability of 23 strains to

survive inside the macrophage was investigated by using the U937 human monocyte cell line.

All species exhibited the ability to attach to epithelial and endothelial cell lines (Caco-2, HBMEC and rBECE4 cells). However, there were differences between species in their attachment to mammalian cell lines; (see Figures 5.1-5.4). For example, *Ent. cancerogenus* and *K. oxytoca* strains showed relatively high levels of attachment to Caco-2 cells compared with *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes* and *Ent. cloacae* strains, (Figure 5.2). While, *Ent. ludwigii* and *Ent. hormaechei* strains showed higher levels of attachment to rBECE4 cells compared to their ability to attach to HBMEC cells (Figures 5.5 and 5.7). This finding is a clear indication of the potential of these bacteria to cause disease.

When the pathogen attaches firmly with the host surface, it then starts to penetrate deep into the host. As it infects the host, the cycle goes on. Although a pathogen might possess an enhanced attachment rate, this may not be the case for invasion. Similar results were obtained in this study. Initially, all *Ent. hormaechei* were found to invade Caco-2 cells. Whereas *Ent. cancerogenus* strains 1350, 1077, 1037, 806 and 1360 and *K. oxytoca* strains 1356 and 1079 which were observed to have highest attachment rates, did not imply the same in invasion rate. The invasion rate was found to be maximum for strain 1053 (Figures 5.1 and 5.3).

In earlier study, the organism *K. oxytoca* has been linked with various conditions such as septicaemia, meningitis, gastrointestinal disease, soft tissue and even in the intravenous region where the host was either immuno-compromised or with conditions that favour pathogen infection conditions (Green *et al.*, 2009). Virulent *Ent. hormaechei*, which has the ability to invade into innermost tissues, resulting in systemic infection has been reported by Townsend *et al.* (2008a). This invasive process by *Ent. hormaechei* also includes gut epithelial cells. Therefore, the pathogenicity and virulence of strains can be determined by the ability of the strain to invade through the gut barrier.

Moreover, in addition to determine the potential pathogenicity of strains, the ability of certain strains to survive inside human macrophages was measured and this included *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca*. Five strains, including three of *Ent. hormaechei* (790, 1038 and 798) and

two of *Ent. cloacae* (779 and 789), were able to survive inside macrophages up to 48 hours. Among the investigated strains, *Ent. cloacae* 779 strain showed signs of a decrease in persistence after 24 hours, whereas *Ent. hormaechei* 798 strain showed the maximum persistence of 48 hours in macrophages after which later on they were killed (Figure 5.9). The remaining three organisms, namely *Ent. ludwigii, Ent. aerogenes* and *K. oxytoca*, were destroyed by macrophages.

In this study, there was a wide variation between strains in intra-macrophage survival data, which is plausibly reflective of the levels of pathogenicity in neonates. According to a previous study, the *Sal.* Enteritidis and *Sal.* Typhimurium, commonly causing localized infections in humans, such as mild gastroenteritis, were not able to survive well in human macrophages when tested *in vitro* (Schwan *et al.*, 2000).

To determine the pathogenicity, the invasive capability through epithelial or brain endothelia and persistence of the bacteria inside macrophages were compared for *Ent. hormaechei* strains 790, 798 and 1038. The mentioned strains could survive in macrophages for extended periods and also had the capability to invade the monolayer of three cell lines (Caco-2, HBMEC and rBECE4 cells) (Table 5.1, Figures. 5.3, 5.6, 5.8 and 5.9). These results substantiate the initial claim. This could also be the factor that aids the bacteria to penetrate the nearly unbreakable barrier, that is, the blood-brain barrier and escape from the host's immune response, and to disseminate.

The aforementioned results corroborate the previous study of Townsend *et al.* (2008a), which investigated the *Cronobacter* spp. outbreak in NICUs in France. That study highlighted the ability of the *Cronobacter* spp., to attach and invade the Caco-2 human epithelial cell barrier. They were found to cross the rat brain capillary endothelial cells and persist inside macrophages for 48 hours. Half of the neonates exhibited clinical symptoms. This places more emphasis on the features which are used to evade the host's immune response and are able to spread inside hosts.

Enterobacteriaceae were also examined to establish any pattern in adherece to the surface of Caco-2 cells. The strains *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae* and *K. oxytoca* were found to adhere to the Caco-2 cells, whereas no definite pattern could be observed. Therefore, it can be concluded

that the bacteria did not adhere with a definite pattern with the Caco-2 human epithelial cells and do nevertheless invade (Table 5.2, Figure 5.10 A, B and C).

The other virulence factors which were studied were the ability to produce haemolysins and siderophores, serum sensitivity and antimicrobial susceptibility. Haemolysin is a toxin produced by the bacteria which also determines its pathogenicity. According to the previous study, α-haemolysin is more virulent among the three and it is expressed by various extra-intestinal pathogenic bacteria including *E. coli* (Balsalobre *et al*., 2006). Among the bacteria studied, except *K. oxytoca* strain 832, all the other strains were found to be α-haemolytic on sheep blood and 17 strains were β-haemolytic on horse blood.

Similarly, Ring *et al*. (2002) have shown that β-hemolysin is an important factor for the sepsis induced by group B *Streptococcus* spp., resulting in liver failure and death in serious cases. Therefore, these results imply that the strains have ability to produce both α-haemolysin and β-hemolysin, which may lysis the RBC cell membranes and cause disease in the neonates.

Another virulence trait of bacterial sensitivity to normal human serum was investigated. Serum resistance was observed in all the strains of *Ent. ludwigii, Ent. hormaechei, Ent. aerogenes, Ent. cloacae,* and *K. oxytoca*, whereas *Ent. ludwigii* strains 1351 and 1439 and *Ent. hormaechei* strains 1053 and 1075 showed intermediate resistance to human serum. This is in agreement with the previous reports which claim that the most common trait of meningococci isolated from blood or cerebrospinal fluid is serum resistance (Figueroa and Densen, 1991). Clinical isolates of *V. vulnificus* showed serum resistance (Bogard and Oliver, 2007). *Ent. hormaechei* strains isolated from different neonates during a nosocomial outbreak in a California hospital were able to survive in human serum (Townsend *et al.*, 2008b).

Next on the list of virulence factors is the requirement for the element iron, which is important for the growth and development of the bacteria that cause the infection (Braun and Winkelman, 1987). This can be identified based on the ability of the bacteria to produce siderophores, a protein which helps in the uptake of iron into the bacteria. In this study, all the bacteria were observed to produce active siderophores. Apart from siderophore production, in the present scenario, molecular characterization was also performed to confirm the pathogenicity of the infecting

strains (Wilson *et al*., 2002). Genes that are responsible for the uptake of iron, *viz*. the *irp1, irp2, fyuA* were detected in HPI, the genetic and structure organiation of HPI are presented in section 1.11 Figure 1.3. Yersiniabactin, a siderophore produced by *Yersinia* spp., is also a factor which confers virulence to it (Schubert *et al*., 2000).

Johnson and Stell (2000) have shown that the genes for urosepsis, *fyuA,* is highly prevalent in virulent *E. coli.* Moreover, *Yersinia* HPI can be used as a marker for the detection of *E. coli* and other *Enterobacteriaceae* which cause extra-intestinal infection (Schubert *et al*., 2002). In this study, the *fyuA* gene was identified in all *Ent. aerogenes* strains, *Ent. cloacae* strains, 4/6 *Ent. ludwigii* strains, 1/8 *Ent. hormaechei* strains, 4/6 *K. oxytoca* strains, whereas the other two genes under investigation were not detected in any of the strains in the study. The fascinating aspect of this study is that some strains of *Ent. ludwigii*, *Ent. hormaechei*, and *K. oxytoca* were able to synthesize siderophores, where no genes previously reported for siderophore production (*irp1*, *irp2* and *fyuA*) could be detected genetically. This shows that the organism has gained another siderophore systems such as pyochelin or anguibactin

lack the hms locus and therefore are pigmentation negative but do carry a 45-kb stretch of chromosomal DNA comprising the irp1-irp2 and fyuA genes.

In addition, all strains of *Ent. ludwigii*, *Ent. hormaechei, Ent. aerogenes, Ent. cloacae,* and *K. oxytoca* have acquired resistance to a broad spectrum of antibiotics. One of the noteworthy results found was that three strains of *Ent. hormaechei* (795, 798 and 860) which exhibited resistance to aminoglycoside, penicillin, tetracycline, chloramphenicol, quinolones and cephalosporins 1, 2 and 3 and were found to be sensitive for carbapenems. Furthermore, most strains showed high levels of cephalosporins resistance in the absence of ESBLs producing genes, which may be because of the hyper production of chromosomal AMPC. AMPC β-lactamases are bacterial enzymes which are clinically important that hydrolyse 3rd generation extended spectrum cephalosporins and cephamycins causing resistance to these types of antibiotic (Jacoby, 2009).

These findings were in agreement with the previous study of Hurrell *et al.* (2009b), who reported the isolation of *Ent. hormaechei* from the surface of enteral feeding tubes at QMC and NCH. Most of the isolates were resistant to even the 3rd generation

cephalosporins, ceftazidime and cefotaxime and were also found to be ESBL-positive. This shows that the neonates were exposed to the strains which were highly resistant to antibiotics.

Hemolysin production may also be associated with the other factors of virulence such as serum resistance and siderophore secretions which may result in neonatal infection.

Finally, in accordance with table 5.1, six isolates can be categorized as high risk as they enhance the chance of infecting the neonates through the contaminated EFT administered infant formula. Those are *Ent. hormaechei* strains 790, 798, 1038, 1053 and 1075 and *Ent. ludwigii* strain 1439.

The risk factor in this analysis was ascertained based on the capacity of the strains under investigation to invade through various mammalian cell lines (Caco-2, HBMEC and rBECE4 cells) and their persistence in the human macrophages. The *Ent. hormaechei* strain 1053 exhibited a relatively high invasive capability in comparison with the other strains, *Ent. hormaechei* strains 790, 798, 1038 and 1075 and *Ent. ludwigii* strain 1439, which exhibited less invasive capability in Caco-2 cells.

HBMEC and rBECE4 cells were used as model systems to investigate the potential of the strains to cross the brain capillary endothelial cell barrier. For three strains of *Ent. hormaechei*, (789, 1053 and 1075), a similar invasive rate was observed in both the cell lines, yet this was not the case of *Ent. ludwigii* strain 1439. The *Ent. ludwigii* strain 1439 exhibited higher levels of invasion in rBECE4 cells rather than HBMEC cell line. The strain was isolated from a neonatal meningitis case at QMC, Nottingham.

There is also a chance that the strain could have infected the neonate as the skull was opened for a brain surgery. However, since this study aimed at evaluating the virulence of the bacteria that cause the disease with the clinical symptoms *in vitro*, it may be a better indication that the bacteria is potentially virulent and can potentially cause meningitis, because of the ability of the strains *Ent. hormaechei* to invade through the HBMEC and rBECE4 cell line barrier.

Chapter 7:CONCLUSION AND FUTURE WORK

7.1 CONCLUSION

The present study indicates the potential exposure of neonates to particular genotypes of strains due to heat tolerance, biofilm formation and acidic pH survival. The study also attempted to describe whether those genotypes have a greater propensity to invade neonatal intestines and invade the brain blood barrier and to evade the immature immune system. The key findings of this project are listed below.

- Twenty-one EFT and 3 MBM pulsotypes of *Enterobacteriaceae* were associated with contaminated neonatal enteral feeding tubes and mastic human breast milk, respectively. This indicates that infants are susceptible to colonisation by *Enterobacteriaceae.*
- The same pulsotypes were spread among the enteral feeding tubes of infants in the same NICUs, indicating the same origins, such as: environment, milk, carers or healthcare equipment.
- Genotypic identification of bacterial strains is more accurate than the phenotypic technique and the profiling of bacteria by PFGE used in this study is a suitable technique for studying strain relatedness.
- Most strains were able to survive at 55ºC for >30 minutes and this indicated that neonates might have been exposed to these organisms either from breast milk or from reconstituted infant formula.
- Strains belonging to the same pulsotypes (Enc1, Enc2, Enc3, Ebh6 and Kc3 pulsotypes) by PFGE analysis showed the same death rate at 55ºC. These clonal strains may have the same source of origin which possibly contaminated different feeding tubes. Similarly, the MBM strains probably were isolated from the same mother or breast pomp. There is also a possibility of ingestion of these strains by neonates where they could have been exposed to the contaminated milk or the infant formula.
- Biofilm formation on plastic materials was influenced by incubation temperature and type of formula in all tested strains.
- The quantity of biofilm was high in casein-based infant formula compared with whey- and soya-based infant formula in all strains. These observations

may indicate the importance of the nutrient type present in PIF for biofilm formation.

- Most strains produced capsule material at 37ºC. This result is strong evidence that body temperature may play an important role in producing capsule material.
- The ability of organisms to survive at pH 3.5 is an indicator that these organisms may pass through the stomach and colonise the intestine which may cause human illness.
- All strains showed an ability to attach Caco-2, HBMEC and rBCEC4 cell lines.
- Many strains invaded Caco-2, HBMEC and rBCEC4 cell lines and persisted and replicated within macrophages which may play a significant role towards morbidity and mortality in NICUs.
- Most *Ent. hormaechei* strains were able to invade HBMEC cell lines at high rates compared to *Ent. ludwigii* strains.
- Many of the *Ent. hormaechei* strains were able to survive within macrophages compared to the rest of the strains.
- The adhesion of bacteria is significant for its ability to cause infection in neonates. All strains did adhere to Caco-2 cells and they showed three different adhesion patterns; aggregative, diffused and localized adhesions. Some strains belonging to the same pulsotype showed the same adhesion patterns. This is an indicator that the neonate was exposed by the same clone which could be from the same source.
- The detection of haemolytic activity, serum resistance and siderophore production in all strains in this study indicate an increased risk to neonates.
- Three of *Ent. hormaechei* strains showed resistance to even the 3rd generation cephalosporins, ceftazidime and cefotaxime and were ESBL-positive. This illustrates the fact that the neonates were exposed to strains which were highly resistant to antibiotics.

7.2 Suggested preventive measures for the transmission of bacteria among neonates in NICU

- Hand hygiene is considered the most important procedures in the prevention of cross contamination and cross infection by bacteria. Social hand washing should be before starting work in NICU, also after changing the gloves.

- Change gloves during neonate care if moving from patient to patient. If the gloves contaminated body site to either another body site (including nonintact skin, mucous membrane, or medical device) within the same patient or the environment you should remove it.

- The health care should use sterile disposal coat in NICU.
- For the waste, do not touch bin lid with hands and dispose of the waste without de-contaminating hands.

- The EFT should be placed in the neonates less than 24 hours to avoid bacteria colonisation.

- Sterile medical equipment (thermometer or stethoscope) should be use in NICU between potions.

- Sterile breast pump should be used in NICU, nipple and mammary areola should be cleaned with soap and sterile water and then express the milk using sterile-gloves.

- Holding different neonates with same coat and gloves should be avoided.

7.3 Limitations and recommendations for future research

It is important to note the methodological limitations of this study. The limitations include poor information about source of MBM strains because we did not know which was obtained from which mother.

In this study all the isolates from EFT were analysed. However, for comparative isolates should have been obtained from diverse sources in NICU such as nurse, breast pump, powder infant formula, water, surface of preparation area, and medical equipment to determine source of contamination in NICU, as described in Figure 1.1.

Also, there was no clinical infection reported for the infants and also it was a long time (from 2007) between collection of strains and this study.

The potentially of risk to neonatal health from Enterobacteriaceae strains for EFT strains and MBM strains should be studied in UK and Spain separately, as comparative study in each country.

Another important limitation of this study is *Ent. aerogenes* and *Ent. cloacae* were not to test their ability to attach and invest HBMEC and rBCEC4 cell lines because, at the time this study was initiated, these strains were considered as *K. oxytoca*.

On the another hand, there was one cluster, Kp2 pulsotype (Figure 3.6, section 3.3.1.3 was determined in two different hospitals (the KAH and PRH Jordan), this is an unexpected result. Thus this hypothesis needs verification by using a different restriction enzyme in the PFGE. Furthermore, more isolates should have been collected from different source as described in Figure 1.1.

The current study did not examine responsible genes for biofilm formation, It is important to determine the expression of several biofilm genes affected by temperature on biofilm formation on plastic material. Such as, *csgABCDEFG* operon and *mlrA*. Moreover, responsible genes to form biofilms on plastic surfaces as well as alfalfa sprouts and Caco-2 cell lines, such as genes *pgaC,* y*hjN* and *wcaD* should be determined.

Accordind to current study results for the synthesis of the capsular material should determine that if the glucose more importand for producing capsular material than galactose or are they equal sorces to support bacterial capsular formation?

Determination of survival curve of strains a range of at pH 2.5, 3.5 and 4.3 (HCl acidified formula) as this is representative of the neonatal stomach conditions on ingestion (Hurrell *et al.,* 2009b). Besides, evaluated intragastric pH changes and acid reflux in response to feeding in infants with normal gastric emptying (Omari, 2003).

Test the ability of organisms to metabolise sialic acid after passing through the blood–brain barrier, as this could be an important virulence factor.

Study an apoptosis due to *Ent. hormaechei,* as this is an important mechanism contributing to the development of diseases.

Finally, molecular methods for detecting of carbapenem resistance genes such as bla_{KPC} gene. Carbapenem resistance among *Enterobacteriaceae*, in particular among *Klebsiella pneumoniae* and *Escherichia coli*, is an emerging problem worldwide.

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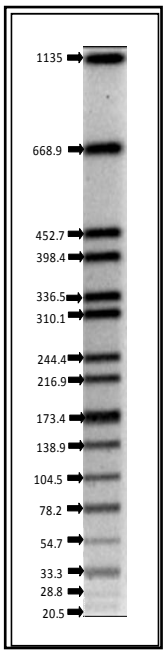
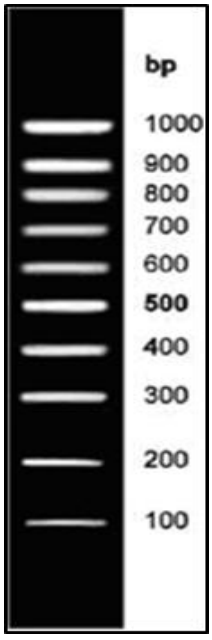
APPENDICES



Appendix 1: *Salmonella* serotype Braenderup reference standard (H9812), approximate band sizes in kilobases shown restricted with *XbaI* and run under the PFGE standardized conditions (Hunter *et al*., 2005).



APPENDIX 2: 100 bp PCR sizer ladder.

Appendix 3: PFGE cluster analysis of *Ent. hormaechei* isolated from EFT (UK).

Strain no.	Source	Date of isolation	Neonate	PFGE cluster
790	NCH	16/01/2007	4	U
795		30/01/2007	17	Ebh6
797			17	
798			18	
799			18	
800			19	
801			20	
802			21	
803			22	
979		16/02/2007	36	Ebh1
980			36	
981			36	
983			36	
986			36	
987			36	
993			36	
997			36	
856		17/04/2007	50	Ebh4
859			51	
860			52	
861			52	
862			52	
863			52	
960		23/05/2007	83	U
1044	QMC	16/10/2007	115	Ebh7
1052			117	Ebh2
1033			110	
1034			110	
1035			110	
1074			117	
1032			110	Ebh3
1053			117	
1075			117	
1027			108	Ebh5
1038		22/10/2007	113	Ebh7
1039			113	
1040			113	
1066		12/11/2007	126	Ebh5
1067			126	
1081			126	
1084			126	
1068		19/11/2007	128	
1069			128	
1089			128	

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Ebh1- Ebh7 indicates PFGE cluster groups, U: Unique.

Appendix 4: PFGE cluster analysis of *Ent. cancerogenus* isolated from EFT(UK).

Strain no.	Source	Date of isolation	Neonate	PFGE cluster
782	NCH	16/01/2007	7	Ebc3
783			8	
781		17/01/2007	6	
784		23/01/2007	9	
806		06/02/2007	24	U
807			24	Ebc3
808			24	
810			26	
814		11/02/2007	27	
815			27	
816			27	
817			27	
818		13/02/2007	28	
824		16/02/2007	36	
982			36	Ebc2
985			36	
988			36	
989			36	
992			36	
994			36	
996			36	
998			36	
990		17/02/2007	37	
991			37	
825			37	Ebc3
826			37	
827		20/02/2007	33	
828			33	
829			33	
831			33	
834			35	
957		22/05/2007	81	Ebc5
966			86	
969			87	
972			87	
842		20/03/2007	42	Ebc3
845		02/04/2007	45	Ebc1
847			45	
848		10/04/2007	48	
850			48	
851			48	
907		24/04/2007	58	Ebc2
908			58	
909			58	
1059	QMC	16/10/2007	119	Ebc4
1071			108	
1037			112	Ebc6
1042		22/10/2007	128	Ebc4
1054			118	
1077		31/10/2007	118	U
1088		19/11/2007	120	Ebc6

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Ebc1- Ebc6 indicates PFGE cluster groups, U:Unique.

Appendix 5: PFGE cluster analysis of *Klebsiella* spp. isolated from EFT (UK).

Strain no.	Source	Date of isolation	Neonate	PFGE cluster
778	NCH	23/01/2007	1	Kc2
785			9	
787			10	
788			11	
792			13	
794			14	
832		16/02/2007	36	Kc5
833			36	
830		20/02/2007	33	
839		28/02/2007	39	Kc4
841			39	
976		30/05/2007	89	Kc6
977			89	
999		05/06/2007	94	
1004			94	
1006			94	
1000			95	
1002			95	
1005			95	
1001			96	
1003			96	
1012		12/06/2007	103	
1013			103	
1014			103	
1046	QMC	22/10/2007	116	Kc1
1048			116	
1049			116	
1078		31/10/2007	120	Kc3
1079			121	
1090		19/11/2007	129	Kc1
1091			129	
1092			129	
1093			129	U

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Kc1- Kc6 indicates PFGE cluster groups, U: Unique.

Appendix 6: PFGE cluster analysis of *Ent. cloacae* and *Ent. aerogenes* isolated from EFT (UK).

Species	Strain no.	Source	Date of isolation	Neonate	PFGE cluster
Ent. cloacae	779	NCH	16/01/2007	2	Ebl1
Ent. cloacae	789		23/01/2007	12	
Ent. aerogenes	1058	QMC	16/10/2007	119	Eba1
Ent. aerogenes	1056		22/10/2007	118	

QMC: Queen's Medical Centre. NCH: Nottingham City Hospital. Ebl1 and Eba1 indicate PFGE cluster groups, U: Unique.

Appendix 7: PFGE cluster analysis of *Klebsiella* spp. isolated from EFT (Jordan).

Strain no.	Date of isolation	Source	Neonate	PFGE cluster	Formula milk
1727, 1728	22/05/2011	KAH	16	Kp2	bebelac
1701, 1702	31/05/2011		8	Kp7	neosure
1703			8	Kp8	
1705, 1706, 1707, 1708			9		S26
1704			8	U	neosure
1699, 1700	04/07//2013		7	Kp7	
1689		PRH	3	Kp2	bebelac
1686, 1687, 1688			3	Kp3	
1691, 1692			4	Kp9	
1690			4	U	
1725, 1726	06/07/2011		15	Kp2	
1722, 1723, 1724		KAH	14		
1709,1710, 1711, 1712		PRH	10	Kp6	bebelac/ S26
1717	07/07/2011		12	Kp2	bebelac
1730, 1731, 1732, 1733	09/07/2011		18	Kp5	
1693, 1694, 1695, 1696			5	Kp9	
1697, 1698			6		
1729	15/07/2011		17	Kp5	
1718, 1719, 1720, 1721	23/07/2011		13	Kp2	
1681, 1682			1		
1716			11		
1715			11	Kp6	
1713, 1714			2		
1683, 1684, 1685			2	Kp3	
1736	07/12/2011		19	Kp2	neosure
1734, 1735		KAH	19	Kp10	
1740	10/12/2011	PRH	21	Kp2	S26
1741, 1742, 1743			22		
1737, 1739			21	Kp4	
1738			20		
1752, 1753	12/12/2011		27	Kp1	
1750	15/12/2011		26	Kp2	
1744, 1745			23	Kp1	
1746, 1747			24		
1748, 1749			25		
1751			26		
1754	20/12/2011		28		
1756			28	Kp2	

KAH: King Abdulla Hospital. PRH: Princesses Rahma Hospital. Kp1-Kp10: indicates PFGE cluster groups; U: Unique.