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Virulence potential and host response to *Cronobacter sakazakii*

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Declaration

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Abstract

Cronobacter sakazakii is in the *Cronobacter* genus (previously known as *Enterobacter sakazakii*), which consists in total of seven species. *C. sakazakii* strains in the clonal complex 4 (CC4), including sequence type 4 (ST4), have been strongly associated with neonatal meningitis. In recent years, research on this organism has made substantial progress using improved identification and molecular methods including multilocus sequence typing. A number of virulence traits have been proposed but have not been studied to date with respect to detailed aspects of the virulence potential and host response.

Therefore this project compared 34 isolates of *C. sakazakii* made up of clonal complex 4 (CC4; 21 isolates) and non-clonal complex 4 (13 isolates) strains for their virulence potential, and investigated whether CC4 strains have the ability to overcome the host barriers more than the other sequence types. The attachment and invasion of mammalian intestinal and brain cells by these strains were evaluated using colorectal adenocarcinoma epithelial cells (Caco-2), human brain microvascular endothelial cells (HBMEC), and rat brain capillary endothelial (rBCEC4) cell lines. Furthermore, the ability of the organism to translocate through different cell lines, including Caco-2 and HBMEC, was assessed. The project also studied the survival of *C. sakazakii* strains in human macrophages (U937) and human microglial cell lines, and the response of these cells in eliminating the infection as a part of the immune response. In addition, it examined the host response to *C. sakazakii* infection.

C. sakazakii strains were motile except for three strains 1223, 1224, and 680. Moreover, the majority of the strains were able to produce iron siderophores except for strains 6 and 520. Additionally, a group of *C. sakazakii* strains were able to withstand serum-mediated killing, whereas strains 6 and 680 were sensitive. The previous traits are important for bacterial growth and survival inside the host. *C. sakazakii* strains showed the ability to adhere and invade the Caco-2, HBMEC, and rBCEC4 cell lines, especially CC4 strains (Caco-2 0.29%, HBMEC 0.13, rBCEC4 0.02%) that displayed the highest invasion levels compared to non-CC4 strains

(Caco-2 0.16%, HBMEC 0.1, rBCEC4 0.016%), supporting the clinical evidence that it can overcome the intestinal and brain barriers. Furthermore, *C. sakazakii* strains, including CC4 strains, were able to translocate through the intact monolayers of the Caco-2 and HBMEC cell lines, and CC4 strains (HBMEC translocation 4.92%) were higher in translocation compared to non-CC4 strains (HBMEC translocation 1.67%). The translocation through Caco-2 and HBMEC is a crucial sign of their invasiveness. The test isolates were able to survive and multiply inside macrophages and microglia. This process is advantageous for the bacterium to survive within the host and evade the immune system.

The test strains, including CC4 strains, triggered the HBMEC cell line to produce iNOS that could lead to elevated levels of NO production leading to cell line permeability. Additionally, the organism was able to induce apoptosis in HBMEC and microglial cells and two markers were detected, caspase-3 and annexin V. Inducing apoptosis in the blood brain barrier cells and microglia is a major threat to the central nervous system (CNS). A number of cytokines were produced by HBMEC and microglial cell lines as a result of *C. sakazakii* exposure. These cytokines included the pro-inflammatory IL-1β, TNF-α, IL-6, and IL-8 in addition to GM-CSF and the anti-inflammatory IL-10 and IL-4. These might contribute to increased blood brain barrier permeability and host damage.

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Table of contents

List of figures

List of tables

List of abbreviations

ACT	Artemis comparison tool
APR	Acute-phase response
ATCC	American Type Culture Collection
BBB	Blood brain barrier
BPW	Buffered peptone water
Caco-2	Human colonic carcinoma epithelial cells
CAD	Caspase activated DNase
CAS	Chrome Azurol S
CASAD	Chrome Azurol S agar diffusion
CC4	Clonal complex 4
CDC	Centers for Disease Control and Prevention
cfu	Colony-forming unit
CNS	Central nervous system
Cpa	*Cronobacter* plasminogen activator
CSF	Cerebrospinal fluid
DFI	Druggan Forsythe Iversen agar
dH$_2$O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ECACC	European Collection of Cell Cultures
EE	*Enterobacteriaceae* broth
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESBL	Extended spectrum β-lactamase
f-AFLP	fluorescent-amplified fragment length polymorphism
Fig.	Figure
GIT	Gastrointestinal tract
GPA	Gentamicin protection assay
HBMEC	Human brain microvascular endothelial cells
HMGC	Human microglial cells
ICAD	Inhibitor of caspase activated DNase
ICAM-1	Intercellular adhesion molecule 1
ICMSF	International Commission of Microbiological Specifications for Food
IEC-6	Rat intestinal epithelial cells

List of abbreviations

IL	Interleukin
iNOS	Inducable nitric oxide synthase
kb	Kilo-base
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
Mb	Mega-base
MEM	Minimum Essential Medium
MHC	Major histocompatibility complex
ml	Millilitre
MLST	Multilocus sequence typing
mm	Millimetres
MMP	Matrix metalloproteinase
MMPs	Matrix metalloproteinases
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NEC	Necrotising enterocolitis
NICU	Neonatal intensive care unit
nm	Nanometre
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
OmpA	Outer membrane protein A
ORF	Open reading frames
PAI-1	Plasminogen activator inhibitor 1
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pg	Picogram
PIF	Powdered infant formula
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear cells
PS	Phosphatidylserine
PTFE	Collagen-coated polytetrafluoroethylene
rBCEC4	Rat brain capillary endothelial cells
RFUs	Relative fluorescence units
SD	Standard deviation

List of abbreviations

SER	Standard error of mean
siRNA	Small interfering RNA
SOD	Superoxide dismutase
ST	sequence type
TAE	Tris-acetate-EDTA
TEER	Transepithelial/endothelial electrical resistance
Th2	T helper 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSA	Tryptone soya agar
TSB	Tryptone Soya broth
U937	Human macrophage cell line
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
α2-AP	α2 antiplasmin
μl	Microliter

Chapter 1: Literature review

1.1. Background

The *Cronobacter* genus is a member of the *Enterobacteriaceae* family. It comprises a distinct group of Gram-negative bacilli that are catalase-positive, oxidase-negative, non-spore forming, facultatively anaerobic, and motile via peritrichous flagella (Baldwin *et al.* 2009, Kucerova *et al.* 2010, Tall *et al.* 2014). The biotyping of this genus was based on many methods including methyl red production, indole test, and production of gas from D-glucose (Iversen and Forsythe 2004, Baldwin *et al.* 2009). *Cronobacter* was previously known as the species *Enterobacter sakazakii*. It was first defined by Farmer *et al.* (1980), and named to honour the Japanese bacteriologist Riichi Sakazaki. At that time, DNA-DNA hybridisation showed that *E. sakazakii* was 41-54% 'related' to species in two distinct genera, *Enterobacter cloacae* and *Citrobacter freundii*. However, as they were phenotypically and genotypically closer to *E. cloacae*, they were placed in the *Enterobacter* genus (Iversen *et al.* 2007, Joseph *et al.* 2012a).

Additional phenotypic analysis demonstrated that *E. sakazakii* comprises 15 biogroups, and biogroup 1 was the most common. A polyphasic taxonomic study using 16S rRNA gene sequencing, ribotyping, fluorescent-amplified fragment length polymorphism (f-AFLP) and DNA–DNA hybridisation showed that the *E. sakazakii* species consist of at least five genomogroups (Iversen *et al.* 2007, Iversen *et al.* 2008). Subsequently in 2007 the *Cronobacter* genus was first defined, and further revised in 2008 and 2012. Following the new definition of this genus, genotypic analysis, such as multilocus sequence typing (MLST), was used to distinguish between the newly identified *Cronobacter* species. Currently the genus contains seven species including *Cronobacter condimenti*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, and *Cronobacter universalis* (Fig.1.1). *C. sakazakii* isolates represent

72.1% of the total strains of *Cronobacter* genus (Joseph *et al.* 2012a, Brady *et al.* 2013).
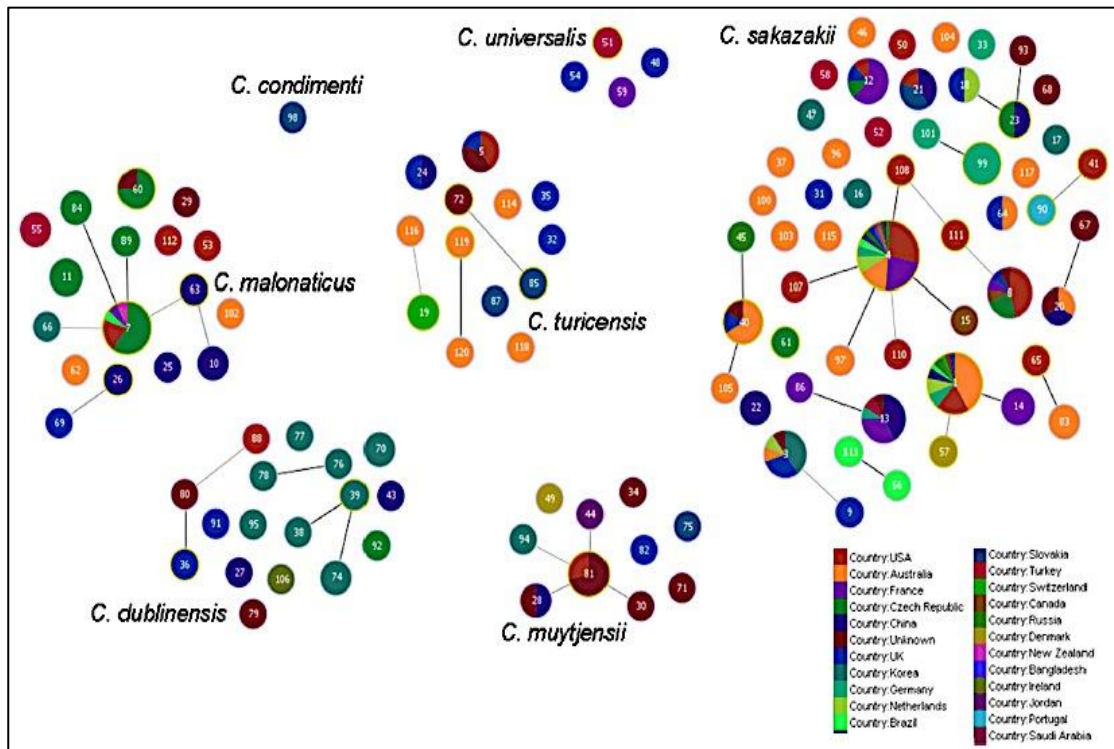
Fig.1.1 Population snapshot of the *Cronobacter* MLST database generated using the GoeBURST algorithm, indicating the clonal complexes and the diversity of the sources of the strains. From Joseph and Forsythe (2012c).

Cronobacter is an emerging opportunistic pathogen that is a concern for the food industry and has been reported as a severe hazard for premature neonates and immunocompromised patients by the International Commission of Microbiological Specifications for Food (ICMSF 2002, Farber and Forsythe 2008). Some *C. sakazakii* infections of neonates have been linked to intrinsically or extrinsically contaminated powdered infant formula (PIF; Caubilla-Barron *et al.* 2007). There are many principles that control risk management of the PIF industry, which should be applied to control the contamination, such as the control of levels in raw materials on receipt, avoiding post-processing contamination, and the application of microbiological criteria such as the number of bacteria in 10 g of PIF. Many batches of formula have been recalled around the world because of contamination (Iversen and Forsythe 2003).

1.2. Source and transmission

As given above, *Cronobacter* can be isolated from PIF. However, it is more widespread and can be found in several environmental and food samples especially from plant-related ingredients. These samples include corn, soy, rice, wheat, cheese, meat, and vegetables (Iversen and Forsythe 2003, Iversen and Forsythe 2004, Baumgartner *et al.* 2009). Furthermore, food might be contaminated via rats and flies such as the Mexican fruit fly and the stable fly (Iversen and Forsythe 2003, Mramba *et al.* 2007). Also *Cronobacter* Spp. can be isolated from follow up formula (Chap *et al.* 2009). PIF is a non-sterile product, and several microbial tests for organisms such as *Escherichia coli (E. coli)*, *Salmonella*, and *Cronobacter* should be applied to each production batch to be compared with specific microbiological measures. Before 2004 the permitted number of *Enterobacteriaceae*, except for *Salmonella*, in PIF was <100 cfu/g. Nonetheless, after the growing concern regarding *Cronobacter* severe infections these microbiological measures changed such that any member of *Cronobacter* genus should not be detectable in 10 g test samples of PIF. The values of these measures are set by the Codex Alimentarius Commission and company-company agreements. *Cronobacter* contamination detection protocol includes pre-enrichment in buffered peptone water (BPW), enrichment in *Enterobacteriaceae* broth (EE), selection on Druggan Forsythe Iversen agar (DFI), and API20E biochemical profiling (Iversen and Forsythe 2003, Forsythe 2005, Commission 2008, Forsythe *et al.* 2014).

In 1960, *E. sakazakii* was isolated from dried milk, which indicates that the organism has been present in these products for decades (Farmer *et al.* 1980, Forsythe 2005). According to Muytjens *et al.* (1988), 14% of 141 PIF samples were found to be contaminated with this organism. These samples were collected from thirteen countries. The range of contamination was between 0.36 and 66 colony-forming units (cfu) per 100 grams (Muytjens *et al.* 1988, Forsythe 2005).

A more recent study, using a specific chromogenic agar to isolate *Cronobacter*, reported that 3 out of 102 PIF samples were contaminated

(Iversen and Forsythe 2004). There is no definitive evidence explaining the route of contamination. It could be through people working in the processing factories or other possible sources. The cleaning process of feeding bottles might be one of the modes of transmission as the bacterium was isolated from a cleaning brush (Forsythe 2005). *Cronobacter* has the ability to grow at a range of temperatures starting from 6 to 45˚C, and form biofilms (Kim and Loessner 2008). Moreover, this organism is highly resistant to heat (72°C) and osmotic pressure. These factors are fundamental for the organism to be successfully transmitted (Nazarowec-White and Farber 2003, Kim and Loessner 2008).

1.3. Epidemiology

Cronobacter spp. was first isolated in 1950 from dried milk powder (*C. sakazakii* NCIMB 8272), whereas the earliest clinical isolate (*C. sakazakii* NCTC 9238) was deposited in 1953 (Farmer *et al.* 1980, Baldwin *et al.* 2009). The first two *Cronobacter* spp. (undefined species) neonatal meningitis cases were reported by Urmenyi and Franklin (1961) when the organism was then still classified as pigmented strains of *E. cloacae*. Biering *et al.* (1989) has reported 3 cases of meningitis due to the same organism that caused one death and resulted in brain damage in the two survivors . Another case reported by Emery and Weymouth (1997) was a 68-year old patient, who had urosepsis and brain atrophy, who died as a result of *Cronobacter* spp. infection. Two other cases of adult infections have been reported by Lai (2001) for a 73-year and 83-year old women who died from biliary sepsis and septicaemia correspondingly. Although infection might occur in adulthood, neonates especially those with low birth weight remain the most susceptible.

Over 100 cases of neonatal *Cronobacter* spp. infections have been published between 2000 and 2008. The mortality rate of these infections was 26.9%. Moreover, the mortality rate of *Cronobacter*-induced meningitis and necrotising enterocolitis (NEC) were 41.9% and 19% respectively (Friedemann 2009). In 2009, CDC reported 2 cases of *C. sakazakii* neonatal infection. The first case showed *C. sakazakii* positive

cerebrospinal fluid (CSF) culture and was discharged with severe brain damage. The other case was for a 7-month male infant. The post-mortem investigation showed the presence of *C. sakazakii* in blood culture (Baumbach *et al.* 2009). *C. sakazakii* was isolated from nine infants in the United States (US) in 2011, five of these isolates were from CSF. One patient died as a result of the infection, and two patients suffered from brain infarction and brain abscess (Hariri *et al.* 2013). Table.1.1 below summarises the cases of *Cronobacter* spp. neonatal infections that were published between 2000 and 2013.

A recent study by Patrick *et al.* (2014) investigated the incidence of the confirmed *Cronobacter* spp. infections and the characteristics of infected individuals in 6 US states. They identified 544 cases in 11 different age groups from 1 day to 100 years, 37% (198) were >70 years and 4% (22) were infants. Urine was the most frequent source of *Cronobacter* spp. isolation by 221 (41%) isolates. It was found that infants were the most susceptible for invasive infections (6 27% of cases) followed by children 1-4 years of age (5 22% of cases), and among adult age groups urine isolates were the most prevalent. The highest incidence rate of invasive infections was among infants 0.49 cases per 100,000 population, followed by patients ≥80 years old (0.33/100,000).

Table.1.1: *Cronobacter* neonatal infection published 2000-2013 adapted from Friedemann (2009).

Year	Number of cases	Infection	Clinical outcome	Country	Reference
1992	1	Meningitis	Death	India	1
1994	10	NEC (7), meningitis (1), septicaemia (2)	Death (4)	France	2
1998	12	NEC (12), bacteraemia (1)	Death (2)	Belgium	3
1998	4	Bacteraemia	Cure	Brazil	4
1998	12	Bacteraemia, urinary tract infection	Unknown	Philippines	4
1999	1	Meningitis	NC	USA	5
2000	2	Meningitis (1), septicaemia (1)	Cure	Israel	6
2001	1	Septicaemia	Cure	USA	7
2001	1	Meningitis	Death	USA	8
2001	2	Meningitis, urinary tract infection	Cure	USA	4
2002	1	Meningitis	Death	Belgium	9
2002	1	Meningitis	Death	Brazil	10
2002	3	Meningitis	Death (1), NC (1)	USA	4
2002	2	Invasive infection	Unknown	USA	4
2003	4	Meningitis (2), bacteraemia (2)	Death (1)	USA	4
2004	1	Meningitis	Death	New Zealand	11
2004	4	Meningitis (2), haemorrhagic colitis (1) conjunctivitis (1)	Death (2)	France	12,13
2004	1	Meningitis	NC	USA	4
2004	1	Invasive infection	Unknown	Netherlands	14
2005	1	Septicaemia	Cure	Slovenia	15
2005	1	Meningitis	NC	USA	4
2006	1	Bacteraemia	Cure	Spain	16
2006	2	Meningitis	Death (2)	Switzerland	17
2006	2	Meningitis	Unknown	Hungary	18
2006	3	Meningitis	NC	USA	4
2007	7	Meningitis (3), bacteraemia (3), NEC (1)	Death (1), NC (1)	USA	4
2007	1	Meningitis	Unknown	Spain	19
2008	1	Meningitis	NC	Japan	4
2008	2	Meningitis	NC (1)	USA	4
2008	1	Meningitis	Cure	Canada	4
2008	1	Invasive infection	Unknown	USA	20
2008	1	Meningitis	Cure	Korea	21
2009	2	Meningitis	Death (1), NC (1)	USA	22
2011	10	Meningitis (5), diarrhoea (2), Unknown (3)	Death (2), NC (2)	USA	23

NEC: necrotising enterocolitis. **NC:** neurological complications. **1:** Ray *et al.* (2007). **2:** Caubilla-Barron *et al.* (2007). **3:** Van Acker *et al.* (2001). **4:** FAO/WHO (2008). **5:** Burdette and Santos (2000). **6:** Bar- Oz *et al.* (2001). **7:** Stoll *et al.* (2004). **8:** Himelright *et al.* (2002). **9:** RASFF (2002). **10:** Barreira *et al.* (2003). **11:** Jarvis (2005). **12:** Coignard *et al.* (2006). **13:** RASFF (2004). **14:** Suijkerbuijk (2008). **15:** Pavcnik-Arnol *et al.* (2007). **16:** Aguirre Conde *et al.* (2007). **17:** Mange *et al.* (2006). **18:** Friedemann (2009). **19:** RASFF (2007). **20:** Friedemann (2009). **21:** Kim and Loessner (2008). **22:** Baumbach *et al.* (2009). **23:** Hariri *et al.* (2013).

Several outbreaks of *C. sakazakii* infections have been reported around the world. In 1994, four infants died in a neonatal intensive care unit (NICU) in France. Three cases were attributed to *C. sakazakii* infections and the fourth was due to *E. cloacae*. They suffered from different forms of infection including NEC and meningitis (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2008). PIF was used to feed the infants through enteral perfusion every four to six hours. *C. sakazakii* strains were isolated from 17 neonates including sputum, faeces, skin, peritoneal fluid, and conjunctivae. API20E was performed to identify the pathogen followed by gas production from glucose as a confirmatory test. Caubilla-Barron *et al.* (2007) examined this incident and used additional techniques to confirm the identity of the pathogens and differentiate between the strains. The techniques used in the aforementioned paper included 16S rRNA gene sequence analysis, and pulsed-field gel electrophoresis (PFGE). There were three different pulsetypes isolated from neonatal samples and PIF. Pulsetype 2 (strains 695, 701, and 767) was linked to three fatal cases (Table.1.3). Hence, this pulsetype was considered more virulent than the others. The antibiograms of strains 695, and 767 exhibited extended spectrum β-lactamase (ESBL) patterns, which might be acquired through horizontal transfer from other *Enterobacteriaceae*, since it was not found in the other strains of pulsetype 2 (Caubilla-Barron *et al.* 2007).

A follow-up study was conducted by Townsend *et al.* (2008) to investigate the *in vitro* virulence ability of the stains recovered from the French NICU outbreak. All strains from each pulsetype showed significant attachment and invasion to Caco-2 cells, at different levels. In addition, after 24 hours incubation period within U937 macrophages, a high number of *C. sakazakii* strains were recovered indicating that they were able to persist inside these cells. Pulsetype 2 (strains 695, 701, and 767) from fatal NEC and meningitis, and pulsetype 4 (strain 716) from PIF showed higher intracellular survival than the other pulsetypes strains. Within the outbreak there was one death due to meningitis. Thus, rat brain capillary endothelial cells (rBCEC4) were utilised to examine the ability of *C. sakazakii* strains to invade these cells, which comprise a part of the blood brain barrier (BBB). Strain 767 from pulsetype 2, which was isolated from

the fatal meningitis case, showed remarkable invasion of rBCEC4. The study suggested that the ability of the organism to invade intestinal epithelial cells is a strong marker of its virulence. Moreover, the organism might be able to overcome the host immune system by surviving and replicating within macrophages. In addition, *C. sakazakii* showed the ability to invade capillary endothelial cells, which indicates that it might gain access to the brain by passing through the normally impassable BBB using specific virulence mechanisms (Townsend *et al.* 2008).

In 1998, 12 neonatal NEC cases were reported at the NICU of Ziekenhis Vrije University hospital in Belgium. All neonates had low birth weight and they were fed orally with infant formula prior to the development of NEC. Surgical intervention was required for 4 neonates suffering from NEC stage III. *Cronobacter* spp. was isolated from 6 out of the 12 patients, and there was a significant correlation between the development of NEC and the consumption of the PIF. It was reported that the molecular typing using arbitrarily primed PCR (AP-PCR) confirmed the similarity between the PIF isolates and three patient isolates (van Acker *et al.* 2001). Another outbreak has been reported by Bar- Oz *et al.* (2001) and Block *et al.* (2002) in Hadassah University Hospital in Israel. *C. sakazakii* was isolated from blood and CSF samples of a baby girl who had brain infarction developing through liquefaction, necrosis, and cavitation. The patient had mild neurological problems and was discharged with two ventriculo-peritoneal shunts at age 3 months. Another patient who had upper gastrointestinal haemorrhage showed positive blood culture for *C. sakazakii*. The organism was also isolated from stool samples of three additional asymptomatic patients.

The previously reviewed cases and outbreaks highlight the significant consequences and the severe outcomes that can be caused by such an organism. Therefore, understanding the behaviour of this organism is important and might provide further insights on the mechanism by which *C. sakazakii* acts and thus will be discussed in the following section.

1.4. Necrotising enterocolitis (NEC)

NEC is the most common gastrointestinal condition in NICU. It is the disease that primarily affects premature infants. Moreover, it is a common cause of death among 20-40% of neonates who have the disease and require surgical intervention (Holman *et al.* 2006). The earliest cases of NEC in the United States were reported in the early 1960s. The estimated fatality rate ranged from 10% to 50% among NEC cases. This disease has different stages NEC I, II, and III (Table.1.2), and it could lead at its final stages to marked abdominal distension, deterioration of vital signs, septic shock, gastrointestinal haemorrhage, and intestinal failure (Bell *et al.* 1978, Henry and Lawrence Moss 2010, Iben and Rodriguez 2011).

Although NEC is a well-recognised medical condition, the pathogenesis and aetiology of it remain poorly understood. This disease is multifactorial, and therefore the attempts to design an experimental model or early diagnostic measures were unsuccessful (Iben and Rodriguez 2011). The factors involved in NEC pathogenesis include bacterial colonisation, intestinal injury, cytokine production, and NO toxicity (Hackam *et al.* 2005, Henry and Lawrence Moss 2010, Iben and Rodriguez 2011). It was found that interleukin-1β (IL-1β) plays an important role in NEC by stimulating the production of matrix metalloproteinase (MMP), which contributes in the degrading of the extracellular matrix. Moreover, the pathological specimens of NEC patients showed intestinal cell apoptosis, production of tumour necrosis factor-α (TNF-α) and IL-8, and the expression of iNOS. The excess production of iNOS induces a high production of NO that leads to cellular damage and intestinal barrier failure. Additionally, the cytokine production that is triggered by bacterial components, such as lipopolysaccharide (LPS), might be involved in the disruption of the tight junctions leading to bacterial translocation. Furthermore, LPS of the Gram-negative bacteria promotes the release of NO and IFN-γ that are signaling molecules, which might cause inhibition of intestinal restitution (Petrosyan *et al.* 2009, Henry and Lawrence Moss 2010, Iben and Rodriguez 2011).

The hypothesis regarding NEC pathogenesis was proposed by Hackam *et al.* (2005) and Iben and Rodriguez (2011). Under perinatal stress such as systemic hypoxia or respiratory distress, the premature infant undergoes a period of intestinal ischemia that results in mucosal injury. Once PIF feeding is introduced to the neonate, the pathogenic enteric bacteria colonise the intestinal mucosal surfaces. These bacteria take advantage of the mucosal injury and translocate through it. This translocation activates macrophages and other cells to produce several inflammatory mediators that could lead to systemic sepsis and then NEC.

A wide range of enteric pathogens are recognised as causative agents of NEC, including *Cronobacter.* It was proposed by Hunter *et al.* (2009) that the organism plays a major role in initiating the onset of NEC. They showed that oral feeding of *Cronobacter* spp. could induce NEC in a rat pup model. Furthermore, it has been demonstrated that the attachment of the bacterium to the enterocytes of the infected animal led to enterocyte apoptosis. Nonetheless, the mechanism by which *Cronobacter* could cause apoptosis of the intestinal epithelial cells is still unknown. They suggested that NO is a vital mediator in *Cronobacter*-mediated NEC, and its toxic metabolite $ONOO^-$ might contribute in the apoptosis induction of rat enterocytes (Hunter *et al.* 2009).

The study by Kim and Loessner (2008) that utilised colon originated human Caco-2 cells, showed that *Cronobacter* can invade human intestinal cells. The entry of the organism into Caco-2 cells might be receptor mediated, and the invasion process depends on bacterial *de novo* protein synthesis. Moreover, the organism can disrupt the tight junctions of the cells, which are important in host cell polarity and prevent molecules from passing freely through the gaps between cells. This disruption requires actin microfilaments, and in turn facilitates and enhances the invasion mechanism. Cytochalasin D (CyD) has the ability to prevent G-actin polymerisation and disrupt F-actin in a concentration-dependent manner. This might inhibit bacterial entry or movement that requires F-actin. It was shown that the invasion of *Cronobacter* was increased in CyD-treated Caco-2 monolayers. This invasion was due to the

disruption of the tight junctions that requires, in the case of *Cronobacter*, actin filaments (Kim and Loessner 2008).

Emami *et al.* (2011) has implicated the role of dendritic cells (DC) in *Cronobacter*-induced NEC. The study showed the ability of *Cronobacter* to disrupt the tight junctions of Caco-2 cell line and pass through the monolayers. This translocation depends on three factors including DC recruitment to lamina properia upon infection that is responsible for intestinal barrier dysfunction, TGF-β secretion by DCs that is involved in tight junction disruption and apoptosis induction in enterocytes, and OmpA expression in *Cronobacter* that is important in the pathogenesis of NEC.

As mentioned previously, a number of *C. sakazakii* outbreaks have been recorded worldwide. In 1994 two *C. sakazakii* NECII and meningitis fatal neonatal infections and one severe NECII were recorded at NICU in France (Caubilla-Barron *et al.* 2007). The isolates showed significant attachment and invasion to Caco-2 cell line (Townsend *et al.* 2008). van Acker *et al.* (2001) reported 12 neonatal NEC cases in the NICU of Ziekenhis Vrije University hospital in Belgium who required surgical intervention. Another outbreak was reported by Bar-Oz *et al.* (2001) and Block *et al.* (2002) in Hadassah University Hospital in Israel. *C. sakazakii* was isolated from CSF of a patient with brain infarction and from a blood of another patient with upper gastrointestinal haemorrhage. These outbreaks highlight the role of *C. sakazakii* in causing severe and fatal infections including NEC. From the information acquired from the previous out breaks it was notable that the patients with brain infections did not suffer from NEC and the isolates were able to pass through gut tissues and reach the brain without inducing significant damage. On the other hand, there is no available information about the ability of NEC isolates to cause brain infections. This suggests that some *C. sakazakii* strains might have a tropism to certain cells.

Table 1.2: Modified Bell's staging of NEC. From Brooks *et al.* (2013).

NEC stage	Systemic signs	Radiographic findings	Intestinal signs
Stage I (suspected)	Temperature instability, apnoea, bradychardia	Normal or intestinal dilation; mild ileus	Gastric residuals, occult blood, mild abdominal distension
Stage II A (definite)	Temperature instability, apnoea, bradycardia	Intestinal dilation, ileus, focal pneumatosis	Blood in stools, prominent abdominal distension, absent bowel sounds.
Stage II B (definite)	As above plus mild metabolic acidosis and thrombocytopenia	As II A plus portal vein gas, ascites.	Abdominal wall oedema with palpable loops and tenderness
Stage III A (advanced)	As stage II B plus mixed acidosis, oligouria, hypotension, coagulopathy	As II B plus worsening ascites	Worsening wall oedema, erythema and induration
Stage III B (advanced)	As II A, shock, deterioration in vital signs	As II B plus pneumoperitoneum	Perforated bowel

1.5. Meningitis

Bacterial meningitis is the inflammation of the meninges following an infection of the central nervous system (CNS), which affects the pia mater (the innermost of the three meninges covering the brain and spinal cord) and the arachnoid and subarachnoid space (O'rahilly and Möller 1986, van de Beek *et al.* 2004, Barichello *et al.* 2013). It is one of the important causes of mortality and morbidity around the world, primarily in neonates and children (Grandgirard and Leib 2010), with a mortality rate ranging between 10% and 15% (Gaschignard *et al.* 2011). 30% to 50% of the survivors are left with neurological abnormalities (Dawson *et al.* 1999, Grimwood *et al.* 2000, Kim 2003). Over 7 million deaths were reported between 2000 and 2010 that occurred in children younger than 5 years old, 64% (4.879 million) of these cases were attributed to infectious agents and 5.2% (0.393 million) of them were linked to sepsis or meningitis (Liu *et al.* 2012a, Barichello *et al.* 2013). In neonatal meningitis, the main pathogens that can cause meningitis are *Streptococcus agalactiae* and *E. coli* K1, while in young children and adults the main causes are *Streptococcus pneumoniae* and *Neisseria meningitidis* (Barichello *et al.* 2013).

The BBB is a highly specialised brain endothelial structure and represents a diffusion barrier that is important to biological functions of the CNS. It is composed of brain microvascular endothelial cells that work in concert

with pericytes, astrocytes, and microglia to separate blood components from neurons (Ballabh *et al.* 2004, Zlokovic 2008). The endothelial cells of the BBB have highly intact tight junctions that regulate the passage of different molecules and ions into and out of the CNS. Moreover, they limit the paracellular flux of hydrophilic molecules throughout the BBB. In contrast, small lipophilic substances diffuse freely through plasma membranes (Rubin and Staddon 1999, Kim 2003, Ballabh *et al.* 2004). Bacteria can cross the BBB using transcellular or paracellular mechanisms, or inside phagocytes such as macrophages (Fig.1.2), which is known as the Trojan horse mechanism (Barichello *et al.* 2013).
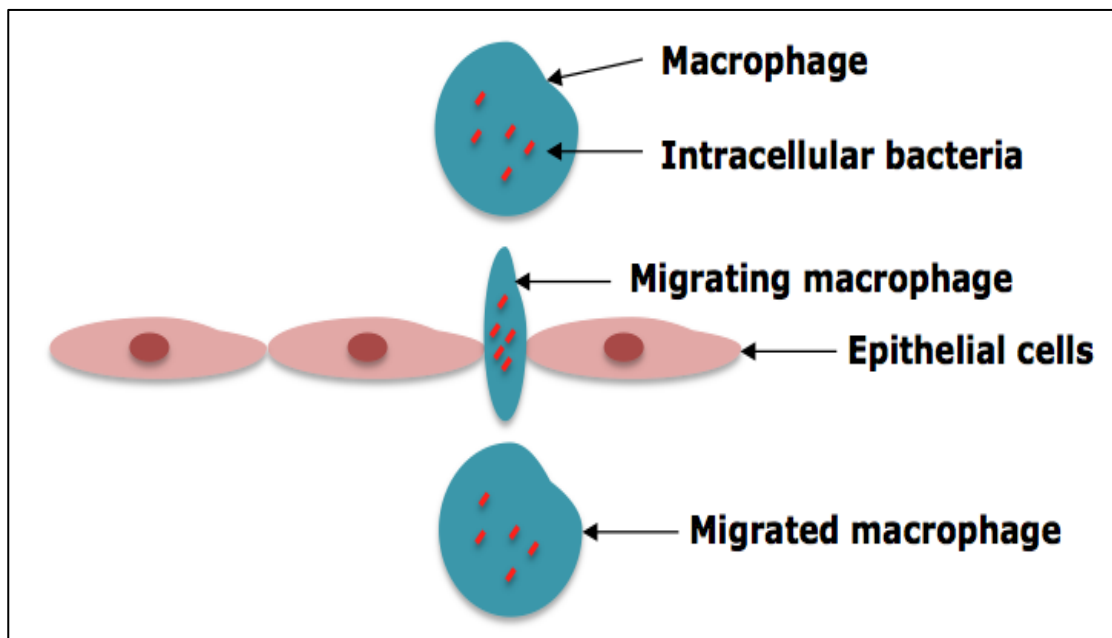
Fig.1.2 Trojan horse invasion mechanism whereby *C. sakazakii* strains might migrate through tissues and blood and avoid host response.

Once bacteria migrate across this barrier, they can multiply within the subarachnoid space concurrently with the release of bacterial components, such as cell wall fragments, which are highly immunogenic and can increase the inflammatory response of the host (Sellner *et al.* 2010, Barichello *et al.* 2013). These products are recognised by pattern-recognition receptors, such as Toll-like receptors (TLRs), of the antigen-presenting cells (Mook-Kanamori *et al.* 2011). There are 11 different TLRs belong to the TLR family that have been defined in human cells (Hanke

and Kielian 2011). Astrocytes express TLRs 2, 3, and 9, while oligodendrocytes express TLRs 2 and 3. Moreover, TLRs 3, 7, 8, and 9 are expressed in neurons, whereas microglia express TLRs 1 to 9 (Mitchell *et al.* 2010). These TLRs (Tables. 1.3-4) are crucial for the initiation of the immune response during meningitis, promoting the production of important inflammatory mediators (Fig.1.3), such as NFκB, which is responsible for the activation of IL-1β, TNF-α, and iNOS that are involved in meningitis pathogenesis (Koedel *et al.* 2000, Kastenbauer *et al.* 2004, Hanke and Kielian 2011).

Table.1.3: TLRs and their ligands in bacterial infection adapted. From Kawai and Akira (2011) and Moresco *et al.* (2011).

TLRs	Ligands	Source
TLR2/1, 2/6	Lipopeptides	Gram-positive bacteria
TLR4	LPS	Gram-negative bacteria
TLR5	Flagellin	Bacterial flagellum
TLR7	RNA	Bacteria
TLR9	DNA	Bacteria

Table.1.4: TLRs functions in CNS injury. From Hanke and Kielian (2011).

CNS insult	TLRs	Pathology
Bacterial meningitis	2, 4 and 9	TLRs are necessary for eliciting maximal antibacterial immunity
Bacterial abscess	2 and 4	TLRs are necessary for eliciting maximal antibacterial immunity
Neuronal injury	2 and 4	TLR2 and TLR4 have been implicated in mediating neuronal death
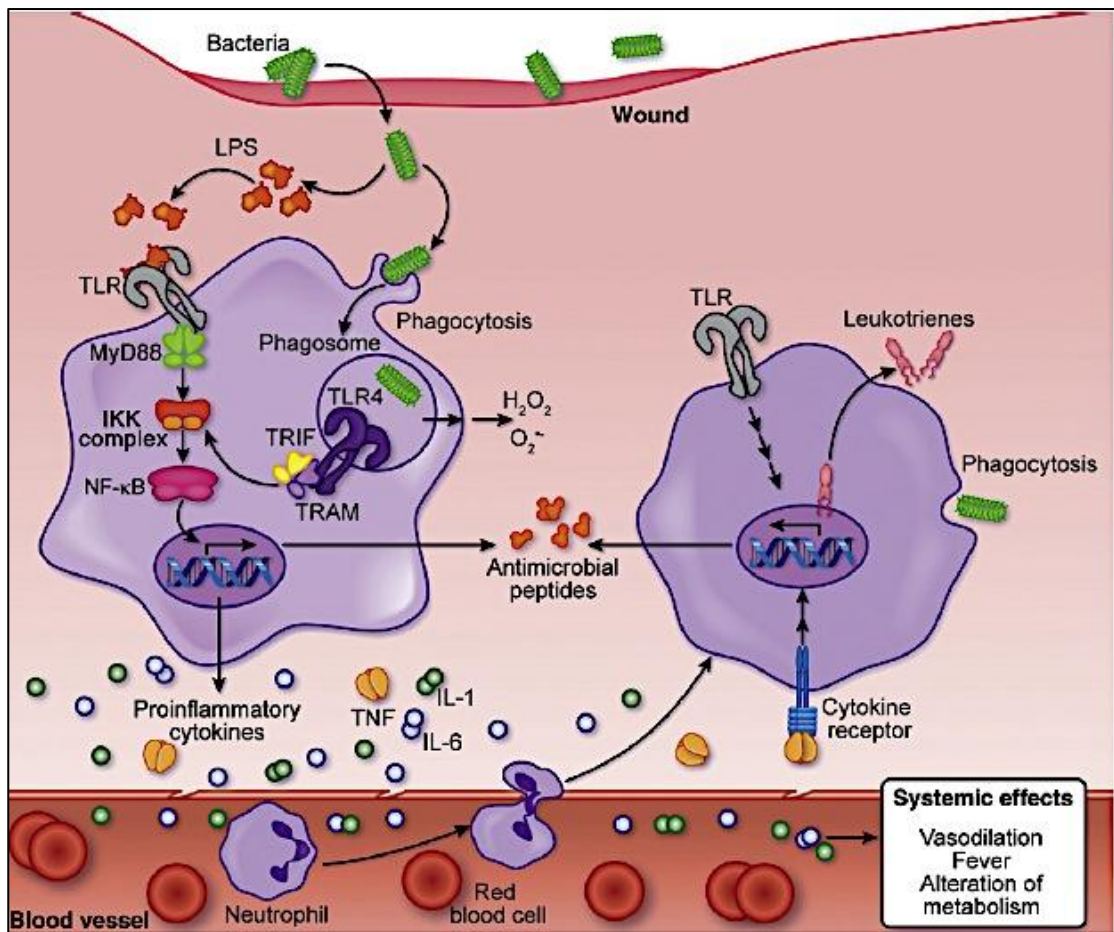
Fig.1.3 TLR intracellular signalling of bacterial infection and inflammation. From Moresco *et al.* (2011).

In addition to the previous mediators, NO and caspases play an important role in BBB permeability during bacterial meningitis. Increased BBB permeability supports plasma leakage into the CNS leading to the development of an inflammatory exudate, cerebral oedema, elevation of intracranial pressure, and alteration of cerebral blood flow (Tunkel and Scheld 1993, Pfister *et al.* 1994, van Furth *et al.* 1996). Moreover, it leads to the migration of white blood cells, especially neutrophils, through the BBB and this process is called pleocytosis (Pfister *et al.* 1994, Braun *et al.* 1999, Scheld *et al.* 2002, Kim 2003). Krebs *et al.* (2005) studied 54 neonates who underwent lumbar puncture, 30 patients had meningitis and CSF samples showed high levels of TNF-α, IL-1β, and IL-6. Additionally, IL-8 was detected in the sera and CSF samples of 45% of tested patients suffering from meningococcal meningitis and bacteraemia.

Furthermore, it was shown in the same study that IL-8 levels peaked after TNF-α and IL-1 induction and at the same time as with IL-6 (Halstensen *et al.* 1993). Furthermore, IL-1β contributes in increasing iNOS induction and subsequently NO production, which in turn disrupts the tight junctions and initiates apoptosis in endothelial cells (Firestein *et al.* 2012). In addition, it was reported that the meningococcus triggers apoptosis in brain endothelial cells via NO that initiates the caspases cascade leading to caspase-3 activation (Schubert-Unkmeir *et al.* 2007). The previously mentioned mediators and mechanisms have a major impact on the BBB that facilitates the passage of bacteria and immune cells leading to severe complications to the host.

Different types of brain cells, including endothelial cells and microglia, have the ability to produce inflammatory mediators (Kronfol and Remick 2000, Scheld *et al.* 2002). Microglial cells were recognised in the late 19th century by the psychiatrist Nissl who named them "rod cells" (Tambuyzer *et al.* 2009). He suggested that they are neuroglia, which have migration and phagocytosis abilities. Moreover, these cells have a fundamental role in iron storage (as ferritin) and detoxification. Later in the 1970s another function was assigned, to blood-borne monocytes that invaded the injured CNS (Barron 1995). Recent reviews indicated that this unique population of cells are CNS resident macrophages, which respond rapidly to the presence of the pathogens and brain damage. Furthermore, they perform phagocytosis, antigen presentation, and are responsible for cytokine secretion. Microglial cells are able to migrate to the injured brain tissues to clear the damaged cells. Some bacterial fractions, such as LPS and DNA, are responsible for the stimulation of microglial cells to produce cytokines (Polazzi and Monti 2010). Therefore, a human microglial cell line was used in this project to investigate the role of microglia in *C. sakazakii*-induced meningitis.

C. sakazakii is responsible for high mortality and morbidity rates due to meningitis. This condition is often linked to severe symptoms such as brain abscess formation and impaired sight and hearing. This kind of

infection will affect the CNS leading to mental and physical abnormalities (Muytjens *et al.* 1983, Mittal *et al.* 2009).

Research reported by Townsend *et al.* (2007b), assessed the inflammatory response against *C. sakazakii* using intracranial inoculation of neonatal rats to describe the progression of brain inflammation. This study showed that *C. sakazakii* strain 2 (*C. sakazakii* ST3; Table.1.7) was able to establish chronic-pattern inflammation and meningitis in 83% of rat neonates, while strain 1 (*C. sakazakii* ST8), which was throat isolate and was not linked to meningitis infection, showed attenuated inflammatory response. Furthermore, it demonstrated that *C. sakazakii* strain 658 (ST1; Table.1.7) led to meningitis in 33% of rat pups. Additionally, strain 57 (*C. turicensis* ST5) induced chronic-pattern inflammation (33%) and meningitis (50%) in rat pups. Neonatal rats that had been inoculated with strain 2 suffered from ventriculitis and meningitis three days post-inoculation. Although strain 658 was linked to a fatal meningitis case, its ability to establish chronic-pattern inflammation and meningitis in rat pups was lower than that of strain 2 that has unknown source and clinical presentation. The organism must cross the BBB to initiate meningitis, and this aspect remains controversial due to lack of a proper *in vivo* model (Townsend *et al.* 2007b).

The gentamicin protection assay was performed to reveal endothelium invasion *in vitro*. This assay showed that *C. sakazakii* strains were able to invade rat capillary endothelial cells and possible cause CNS infection (Townsend *et al.* 2007b). The previous study involved the examination of *Cronobacter* to survive within U937 human macrophage cell line and to induce cytokine production, which highlights the ability of this organism to withstand phagocytosis and activate the immune response. This also suggests that this organism might be able to survive within microglial cells and induce them to produce inflammatory mediators that might contribute to the pathogenesis of meningitis. All strains were able to persist in macrophages for up to 96 hours, however the extent of persistence differed between strains. Strain 2 (*C. sakazakii* ST3; Table.1.7) has the ability of significant replication inside the macrophages while strains 1 (*C.*

sakazakii ST8), 57 (*C. turicensis* ST5), and 84 (*C. dublinensis* ST43) showed a modest level of replication, whereas strain 3 (*C. muytjensii* ST28) exhibited higher sensitivity to macrophage killing among *C. sakazakii* strains (Townsend *et al.* 2007b).

Since macrophages are key regulators of the innate immunity, which trigger a number of inflammatory responses (Fig.1.4), including adaptive immunity, the secreted cytokines were examined using enzyme linked immunosorbent assay (ELISA) following *Cronobacter* infection. Although the levels of TNFα were stable from 6 to 24 hours, the strain NTU84 (*C. dublinensis* ST43) induced secretion of high levels of TNFα. The levels of IL-6 were elevated and the strain NTU84 generated the most robust response. This is an indication of a strong inflammatory response evoked from macrophages in response to the infection. IL-10 and IL-6 expression was detected also in the sera of neonatal rats as a response to *C. sakazakii* strain 2 infection. (Townsend *et al.* 2007b).
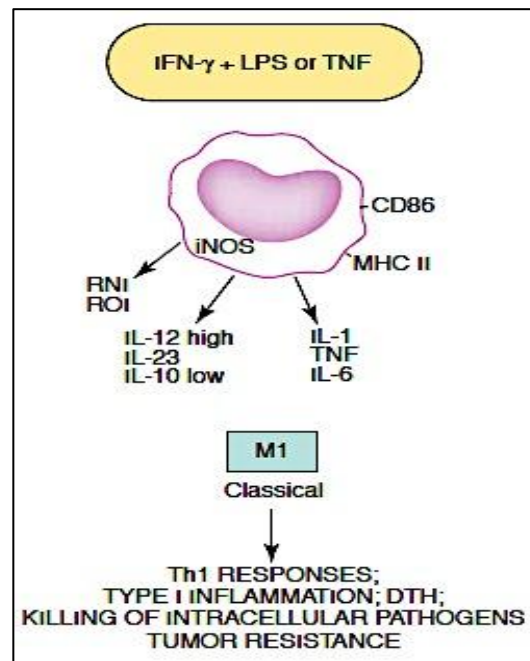
Fig.1.4 Functional properties of activated macrophage showing M1 polarisation that is derived by IFN-Y, LPS, and TNF. From Mantovani *et al.* (2004).

A previous study by Giri *et al.* (2011) examined the ability of *C. sakazakii* strains to invade and translocate through Caco-2 and HBMEC cell lines. It was shown that *C. sakazakii* strains can successfully accomplish this and

that the Caco-2 translocation occurred within 2 hours of incubation and noticeably increased after 4 hours. In addition, strain 1588, which was an environmental isolate, was found to be the most invasive strain for HBMEC cells. Moreover, the organism exhibited the ability of translocating through HBMEC cells whilst *C. sakazakii* strain 1590, which demonstrated low invasion of this cell line, was the lowest in translocation ability. In contrast, strain 1588 demonstrated highest translocation capability, whereas the negative control *E. coli* HB101 was not able to translocate through the intact monolayer (Giri *et al.* 2011). The previous study showed the ability of the organism to invade Caco-2 and HBMEC cell lines and pass through them. Nevertheless, most of the tested strains included in this study were food and environmental isolates.

Citrobacter koseri is a member of the *Enterobacteriaceae* family and is also closely related to *Cronobacter* spp. (Joseph *et al.* 2012c). It is a Gram-negative facultative anaerobic bacillus that colonises the gastrointestinal tract and causes urinary tract infections and intra-abdominal infections. Furthermore, it is a common cause of meningitis in neonates. This organism has the ability to invade brain microvascular endothelial cells, and persist inside the macrophages by avoiding phagocyte killing. In addition, it triggers the inflammatory responses of microglial cells leading to the establishment of a severe *Cit. koseri* CNS infection (Ribeiro *et al.* 1976, Rose 1979, Doran 1999, Pollara *et al.* 2011). Therefore, due to its ability to cause CNS infection especially in neonates and intracellular survival, it represents a convenient positive control to be used in brain cell invasion assays of this project in addition to phagocytosis survival within macrophages and microglia.

1.6. Pathogenicity and virulence

Cronobacter is an opportunistic pathogen, which is associated with a number of clinical presentations in neonates, especially with those who have underlying conditions such as prematurity and low weight at birth. Moreover, the infection is not limited to neonates, it can occur in several age groups with less severity (Caubilla-Barron *et al.* 2007, Patrick *et al.* 2014).

Pagotto *et al.* (2003) indicated that *Cronobacter* isolates are able to produce enterotoxins, and the toxin was lethal to suckling mice. In addition, Townsend *et al.* (2007b) proposed that the organism is able to survive within human macrophages, and penetrate rat capillary endothelial cells. Furthermore, a study by Adegbola and Old (1983) reported that it has the ability to produce mannose-sensitive haemaglutinin associated with type I pilus, which participates in the adhesion process (Kim and Loessner 2008).

It has also been reported that the outer membrane protein A (OmpA) of *Cronobacter* spp. has a role in the colonisation of the gastrointestinal tract (GIT)., This might help in the invasion of human intestinal, which subsequently leads to the survival in the blood, and then invasion of the brain endothelial cells to cause meningitis (Franco *et al.* 2011b). Moreover, it was demonstrated recently that the outer membrane proteins OmpA and OmpX were required for the basolateral invasion of enterocyte-like human epithelial cells by *C. sakazakii* (Kim *et al.* 2010). Additionally, Singamsetty *et al.* (2008) demonstrated that the entry of *Cronobacter* spp. into human brain microvascular endothelial cells (HBMEC) requires OmpA expression and depends on microtubule condensation in these cells. It was shown by Mohan Nair *et al.* (2009) that OmpA of *Cronobacter* spp. is considered as a major fibronectin-binding protein that promotes the invasion of HBMEC of the BBB.

Bacterial cell attachment to surfaces might be followed by growth and biofilm formation (Kumar and Anand 1998). Biofilm refers to immobile communities of bacterial cells attached to each other or to a surface and embedded in polymeric substance produced by bacteria. Biofilm formation following the attachment of the organism to biotic or abiotic surfaces enhances the ability of the organism to resist environmental stress and provides protection against any bactericidal effect (Marshall 1992, Kim *et al.* 2006). It was reported by Kim *et al.* (2006) that *Cronobacter* spp. can form biofilms on enteral feeding tubes and stainless steel. Hartmann *et al.* (2010) have implicated the role of two open reading frames (ORFs), ESA_00281 and ESA_00282, of *C. sakazakii* in the adhesion to Caco-2

cells and biofilm formation. It was also shown in the same study that the flagella of *C. sakazakii* aid the adherence to this biotic surface and contribute in biofilm formation. Flagella are primarily responsible for motility in bacteria. It was shown that *C. sakazakii* mutants of *flhE*, *fliD*, and *flgJ* genes that had lost the ability to express flagella or have shorter ones demonstrated low levels of adherence to Caco-2 cell line and biofilm formation (Hartmann *et al.* 2010).

Nitric oxide (NO) is an endogenously produced molecule that has a critical role in defending against infection. It is a lipophilic and a hydrophilic natural gas that can cross membranes readily. NO has the capacity to react with oxygen and superoxide spontaneously to produce nitrogen and oxygen intermediates to form various antimicrobial intermediates. The latter become biologically significant when the concentration of NO exceeds 1 µM. At such concentrations, reactive nitrogen oxide species (RNOS) could lead to oxidative and nitrosative damage by altering DNA, inhibiting enzyme function, and inducing lipid peroxidation that are responsible for antimicrobial properties. On the other hand, at low NO concentrations (<1 µM) it acts as a signaling molecule that promotes the growth and activity of immune cells (Schairer *et al.* 2012).

NO, which is a short-lived highly reactive molecule, has an essential role in the pathogenesis of the failure of the intestinal barrier in NEC. It is an inflammatory mediator, which can induce enterocyte apoptosis and necrosis and alter the tight junctions (Chokshi *et al.* 2008). NO is produced by three isoforms of NO synthase (NOS). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are expressed at low levels. The last isoform, inducible NOS (iNOS), is not produced under normal conditions. Nonetheless, it is significantly elevated during inflammation leading to high levels of NO production. Clinical samples from infants with NEC demonstrated increased levels of iNOS (Hackam *et al.* 2005, Henry and Lawrence Moss 2010, Iben and Rodriguez 2011). Moreover, as mentioned previously, bacteria can induce endothelial cells to produce NO that collaborates in BBB permeability allowing bacterial cells to migrate to the CNS (Kim 2003).

It was suggested by Hunter *et al.* (2009) that *Cronobacter* stimulates NO production, which leads to apoptosis of rat intestinal epithelial cells (IEC-6) *in vitro* and enterocytes *in vivo*. Overexpression of NO or its toxic metabolite ONOO⁻ may promote gut barrier failure and mucosal injury. The inhibition of NO production by using small interfering RNA (siRNA) to iNOS suppressed *Cronobacter*-induced apoptosis indicating that NO is required for *Cronobacter*-induced apoptosis of IEC-6 cells (Hunter *et al.* 2009).

1.7. Genome studies and identification of putative virulence factors

The first sequenced genome of *Cronobacter* genus was for *C. sakazakii* strain ATCC BAA-894 (658; Table.1.7), and was published by Kucerova *et al.* (2010). Strain ATCC BAA-894 was isolated from a formula tin associated with an NICU outbreak by Himelright *et al.* (2001). The sequence was used for comparative genomic hybridisation (CGH) analysis of physiological and virulence related features against 10 strains represent five different recognised species of *Cronobacter* genus. The sequencing showed that the genome is composed of 1 chromosome (4.36837 Mb) and two plasmids; pESA2 (31kb, 38 genes) and pESA3 (131 kb, 127 genes).

CGH analysis revealed 21 distinctive genes for *C. sakazakii*, which were not found in *C. malonaticus*, *C. muytjensii*, *C. turicensis*, and *C. dublinensis*. These genes encoded two protein clusters involved in pilus assembly, pilin FimA proteins, porin PapC, and the chaperone PapD. Moreover, these genes also included the proteins of phosphotransferase system, a putative sialic acid transporter, N-acetylneuraminate lyase and RelB from a toxin/antitoxin system. In addition, the analysis demonstrated that *C. sakazakii* BAA-894 genome encoded for genes that might contribute in HBMEC invasion including the gene encoding OmpA. Additionally, *cusC*, which is a part of a group of genes encoding a copper and silver resistance cation efflux system, was present. This gene is synonymous to *ibeB* gene that could be associated with the invasion of BBB and can be found in neonatal meningitic *E. coli* K1 (Huang *et al.*

1999, Kucerova *et al.* 2010). The complete cation efflux system *cusA*, *cusB*, *cusC*, *cusF*, and its regulatory gene *cusR* was identified in strains isolated from neonatal infections including strains 701 and 767 (Kucerova *et al.* 2010).

The genes that were found to be shared among three strains linked to *C. sakazakii* infections in NICUs (BAA-894, 701, 767; Table.1.7) were compared with the type strain (ATCC 29544; strain 1; Table.1.7). The latter showed decreased virulence potential in tissue culture studies compared to strains 701 and 767 (Townsend *et al.* 2007b). One hundred and forty four genes were absent in the type strain based on BAA-894 annotation. These genes are involved in oxidative stress resistance, a type VI secretion system, serum resistance, and multidrug efflux components (Kucerova *et al.* 2010, Franco *et al.* 2011b).

As mentioned previously, the whole genome sequencing of *C. sakazakii* BAA-894 showed that the strain carries two plasmids; pESA2 and pESA3 (Kucerova *et al.* 2010). Several virulence gene clusters are encoded on pESA3 including iron acquisition loci; a homologue of an ABC transporter-mediated iron uptake and siderophore biosynthesis system *eit*CBAD operon and a siderophore iron acquisition system *iuc*ABCD/*iut*A operon (Franco *et al.* 2011a). There are two ORFs upstream of the cronobactin gene *iucA*, named *shiF* and *viuB*. The putative protein encoded by *viuB* has a significant similarity to ViuB and YqjH encoded by *Vibrio cholerae* and *E. coli*. These two proteins are accountable for reducing the iron form ferric state to ferrous state leading to the loss of affinity of the ferrous iron for the siderophore. Moreover, *in silico* identification of putative Fur boxes and expression of the genes under iron-depleted conditions suggest that most of these iron transport systems form part of the Fur regulon (Grim *et al.* 2012). By using Chrome Azurol S (CAS) agar diffusion (CASAD) assay, the strains showed the production of active siderophores (Franco *et al.* 2011a). Iron is a cofactor for important enzymes that are involved in many essential cell functions such as electron transfer, cellular respiration, and superoxide metabolism. Iron also is a fundamental element for bacterial pathogenesis. The human body limits iron availability

through iron-binding proteins as a part of the innate immune system and this in turn by reducing free iron levels will not support bacterial growth. In Gram-negative bacteria, during iron starvation conditions, bacteria construct high-affinity iron binding molecules, such as siderophores, to scavenge iron from the environment (Franco *et al.* 2011a). The iron siderophore complexes are taken into the bacterial cell by specific transport systems consisting of an outer membrane receptor, periplasmic binding protein and ABC transporter formed by a permease, and ATPase proteins (Köster 2001, Faraldo-Gómez and Sansom 2003).

It was determined, by using *in silico* analysis, that pESA3 encodes an outer membrane protease related to proteins belonging to the omptin family. These omptins include a group of proteases, which are surface orientated outer membrane proteins expressed by many members of the *Enterobacteriaceae*. Most of the known omptins act as proteases, adhesins, or invasins (Franco *et al.* 2011b). Based on amino acid sequence analysis of these omptins, it has been found that they consist of two groups. The first group is called Pla subfamily, which includes PgtE of *Salmonella* Enteritidis, Pla of *Yersinia pestis*, and PlaA of *Erwinia* spp. The second subfamily is OmpT, which consists of OmpT and OmpP of *E. coli* and SopA of *Shigella flexneri* (Kukkonen and Korhonen 2004, Haiko *et al.* 2009). Pla and PgtE have a role in the conversion of human proenzyme plasminogen to plasmin, inactivation of plasmin inhibitors α2 antiplasmin (α2-AP), and plasminogen activator inhibitor 1 (PAI-1; Kukkonen *et al.* 2001, Lähteenmäki *et al.* 2005b, Haiko *et al.* 2010, Franco *et al.* 2011a).

Plasmin is a broad-specificity serine protease which can activate other proteolytic enzymes, including MMPs that have the ability to degrade the tight junction components of microvascular endothelial cells (Lähteenmäki *et al.* 2005). This function is crucial for plasmin-mediated mechanism of intercellular migration, which allows the bacteria to cross the vasculature into either the peripheral tissue or advantaged compartment such as the CNS. Inactivation of α2-AP by Pla provides uncontrolled proteolysis that contributes in the invasion of the bacteria by altering complement-

dependent killing (Franco *et al.* 2011b). Furthermore, Pla and PgtE are able to degrade some serum proteins, including circulating complement providing the protection of *Y. pestis* and *S. enterica* against complement-dependent serum killing (Sodeinde *et al.* 1992, Ramu *et al.* 2007). The alignment of the amino acid sequence of *C. sakazakii* outer membrane protease with different omptin family members shows that the protease belongs to Pla subfamily. As a result of that, the outer membrane protease was named *Cronobacter* plasminogen activator (Cpa). Cpa of *C. sakazakii* may provide resistance to bactericidal activity of serum through cleaving complement components C3 and C4b beside the activation of plasminogen and inactivation of α2-AP (Franco *et al.* 2011b). According to Joseph *et al.* (2012b), the gene encoding Cpa was found in *C. sakazakii* strains 696 and 701, whereas it was absent in strain 680. Moreover, the other species of *Cronobacter* lacked the presence of this gene except for *C. universalis*.

A study by Joseph *et al.* (2012b) showed that the comparison of three *C. sakazakii* genomes with other *Cronobacter* species revealed 408 ORFs that are unique within this species. Strain 696 and 701 (Table.1.7) were found to have a number of unique regions. *C. sakazakii* strain 680 (Table.1.7) had unique genes encoding a region that is responsible for iron siderophore *fecRABCDE* that is possibly plasmid-borne in addition to arsenate resistance. On the other hand, compared to the reference genome *C. sakazakii* BAA-894, this strain is missing large regions including flagella synthesis genes. Moreover, the *wza* gene cluster that encodes for the exopolysacchride colanic acid was found in all *Cronobacter* strains used in the previous study (Joseph *et al.* 2012b). Furthermore, haemolysin and haemolysin-related genes were found in all *Cronobacter* strains except for *C. sakazakii* strain 701 and *C. malonaticus* strain 507. Furthermore, all *Cronobacter* genomes were found to encode macrophage survival gene *sodA*, and metallprotease *zpx* genes. *C. sakazakii* genomes encoded for important gene cluster *nanAKTRC* that is linked to the uptake and utilisation of sialic acid. Moreover, these genomes encoded for the putative sugar isomerase YhcH. The *nan* cluster was found to be adjacent to the starvation gene homologue *sspA*, hence this cluster might be

responsive to the environmental nutrient levels. (Joseph *et al.* 2012b). In another study by Joseph *et al.* (2013), it was found that *nanAKTRC* and *yhcH* were found only in *C. sakazakii* genomes. The ability of this organism to acquire exogenous sialic acid might have a major role in *C. sakazakii* colonisation of the host. Human body has three different sources rich in sialic acid the gastrointestinal tract, the brain, and human milk. Thus the utilisation of sialic acid could be a virulence factor that could contribute in gut and brain infections, as the bacterium has a history of association with NEC and meningitis infections (Joseph *et al.* 2012b, Joseph *et al.* 2013). Table.1.5 below shows the presence/absence of some important virulence genes in *C. sakazakii* based on Joseph *et al.* (2012b).

Table.1.5: Presence/absence of some important virulence genes in *C. sakazakii*. From Joseph *et al.* (2012b).

Loci	658	701	680	696	Comments
ESA_00140–ESA_00145	+	+	+	+	Encodes most of the proteins that are conserved across different T6SS clusters, including DotU homologue (ESA_00140), Vgr homologue (ESA_00141), and a putative lipoprotein from the VC_A0113 family (ESA_00145)
ESA_02035–ESA_02040	+	+	-	-	Includes Vgr-type protein (ESA_02035), a lipoprotein from the VC_A0113 family (ESA_02038), and other genes homologous to proteins encoded in the T6SS clusters
ESA_02735–ESA_02740	+	+	-	+	Contains genes encoding SciE-type protein (ESA_02736), Vgr-type protein (ESA_02739), and a protein homologous to phage gp7 protein; adjacent to phage
ESA_03887–ESA_03946	+	+	+	+	Longest and most complete cluster; included genes encoding Vgr-type proteins (ESA_03905 and ESA_03917), IcmF-type protein (ESA_03945), DotU-type protein (ESA_03946), ClpV ATPase (ESA_03921), SciE-type protein (ESA_03925), Ser/Thr protein phosphatase (ESA_03927), and Ser/Thr protein kinase (ESA_03920)
ESA_pESA3p05491–ESA_pESA3p05506	+	-	+	+	Plasmid borne; ESA_pESA3p05494 encodes DotU-like protein and ESA_pESA3p05495 encodes a protein with a C-terminal extension with similarity to ompA. ESA_pESA3p05497 encodes a ClpV ATPase and ESA_pESA3p05500 encodes a Vgr-like protein.
ESA_00102	+	+	+	+	Haemolysin activator protein precursor
ESA_00432	+	+	+	+	Predicted membrane protein haemolysin III homolog
ESA_00643	+	+	-	+	Haemolysins and related proteins containing CBS domains
ESA_02810	+	+	+	+	Haemolysin expression modulating protein
ESA_02937	+	+	+	+	Putative haemolysin
ESA_03540	+	-	+	+	21 kDa haemolysin precursor

The previous discussion illustrates how aggressive this pathogen can be and the deleterious consequences that might result from the infection. Although the virulence potential and the pathogenicity of the organism

have been extensively researched over the recent years, none of the studies reported in the literature have examined the host response and its role in the pathogenesis of meningitis. There are still a number of issues currently limiting obtaining the complete picture of the mechanism by which *C. sakazakii* could overcome host barriers and evade immune response. Therefore, further understanding of the factors that might contribute in the severity of the infection and could lead to host damage is required.

1.8. Multilocus sequence typing

A multilocus sequence typing scheme (MLST) of *Cronobacter* spp. has been constructed, initially for *C. sakazakii* and *C. malonaticus*, by Baldwin *et al.* (2009) which now covers all seven *Cronobacter* species and is available online at http://www.pubMLST.org/cronobacter. This typing scheme is based on DNA sequence variation of seven loci; *atpD*, *fusA*, *glnS, gltB, gyrB*, *infB*, and *ppsA* (Baldwin *et al.* 2009, Joseph and Forsythe 2012c).

Joseph and Forsythe (2011) investigated the association between the severity of the infection and the sequence type (ST) profile. A total of 41 clinical isolates of *C. sakazakii* from seven countries were included in the study. The sequence analysis was compared with the *Cronobacter* MLST database. It demonstrated that the test clinical isolates were in 10 of the 25 STs identified for *C. sakazakii* (Table.1.7). Moreover, it showed that 20 strains were ST4, and the others were ST8 (7), ST1 (4), ST12 (3), ST3 (2) and single strains in ST13, ST15, ST18, ST31, and ST41. Nine ST4 strains were meningitis isolates, and five ST4 strains were isolated from CSF samples. However, there were only two meningitis strains from ST1. It was proposed that ST4 is a highly stable clone as it was isolated from seven different countries over fifty years. In addition, the most virulent strains that caused fatal infections, meningitis in particular, belong to ST4. The study concluded that further studies to investigate the clonal nature of ST4 and virulence are warranted (Joseph and Forsythe 2011).

Table.1.6: *C. sakazakii* STs and their sources. From Joseph and Forsythe (2011).

Source	ST1	ST3	ST4	ST8	ST12	ST13	ST15	ST18	ST31	ST41
CSF	2	-	5	-	-	-	-	-	-	-
Blood	1	-	4	-	-	-	-	-	-	-
Trachea	-	-	3	-	-	-	-	-	-	-
Faeces	1	-	2	-	2	1	-	-	-	-
EFT	-	2	-	-	-	-	-	-	-	-
Brain	-	-	1	-	-	-	-	-	-	-
PF	-	-	1	-	-	-	-	-	-	-
Throat	-	-	-	1	-	-	-	-	-	-
Wound	-	-	-	-	-	-	-	-	-	1
Sputum	-	-	-	1	-	-	-	-	-	-
SF	-	-	-	1	-	-	-	-	-	-
AP	-	-	-	-	-	-	-	1	-	-
Unknown	-	-	4	4	1	-	1	-	1	-

EFT: enteral feeding tube.
PF: peritoneal fluid.
SF: spinal fluid.
AP: abdomen pus.

A follow-up study showed that eBURST analysis exhibited 13 single locus variant clonal complexes among the 115 identified STs of *Cronobacter* genus. The clonal complex where strains are identical in 3 or more loci, and *C. sakazakii* is represented in nine of these clades (Forsythe *et al.* 2014). Clonal complex 4 (CC4) contains *C. sakazakii* STs 4, 15, 97, 107, 108, and 109, and as mentioned previously ST4 is the most frequent clinical ST (Fig.1.5). ST 15 has one isolate from a Canadian clinical case. The study revealed the high clonality of CC4 strains and their association with neonatal meningitis cases (Joseph and Forsythe 2011, Joseph and Forsythe 2012c). Another study by Joseph *et al.* (2012d) included 325 strains across the *Cronobacter* genus. It was found that *C. sakazakii* comprises seventeen STs, and ST4 was most prevalent with 78 isolates. It was also shown that *C. sakazakii* was the predominant species from clinical sources, and ST4 was the most prevalent ST of meningitis cases and CSF isolates (Joseph *et al.* 2012d, Forsythe *et al.* 2014).
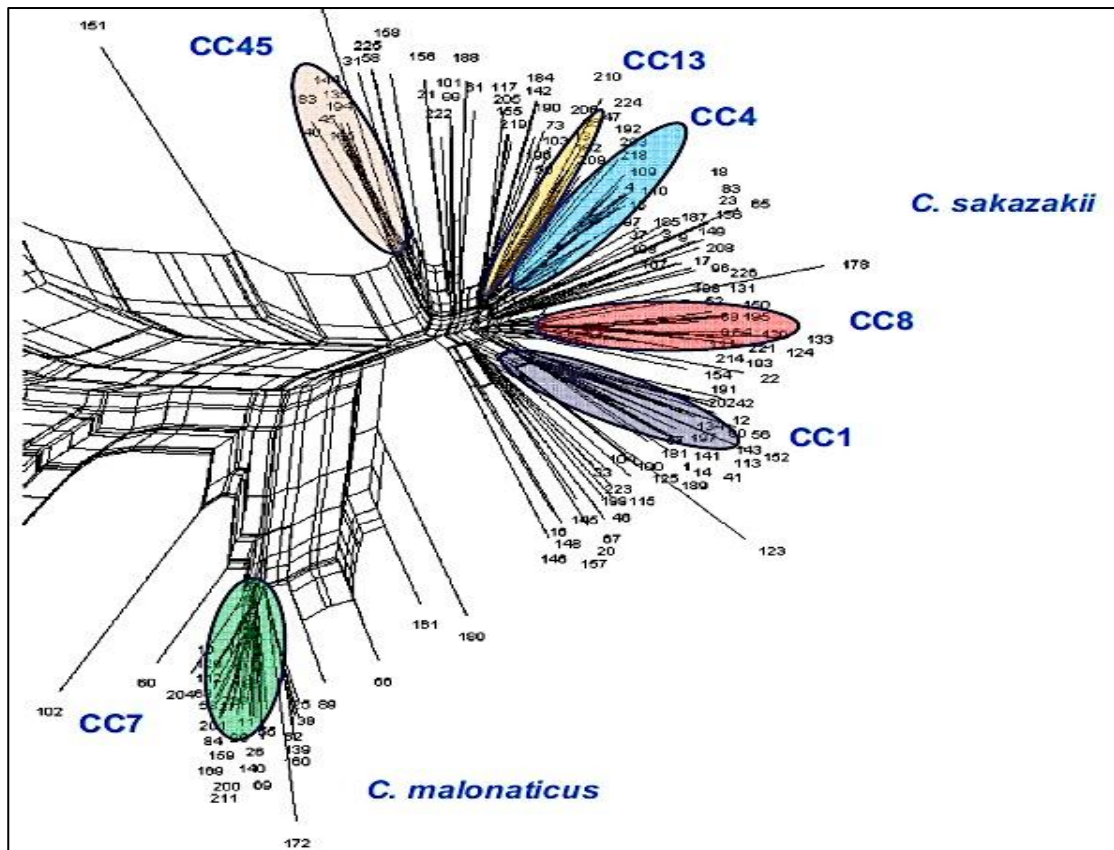
Fig.1.5 *C. sakazakii* and *C. malonaticus* with major clonal lineages highlighted using 7-loci Splits Network of 298 sequence types from the *Cronobacter* genus. From Forsythe *et al.* (2014)

Hariri *et al.* (2013) investigated 15 *Cronobacter* isolates received from the Centers for Disease Control and Pervention (CDC), which were collected during 2011. Most (14) were *C. sakazakii* and 1 *C. malonaticus* meningitis isolate. Nine samples were clinical isolates from neonates or infants. The CSF isolates (5) were either ST4 or within CC4. The study concluded that this investigation supports the conclusion that *C. sakazakii* CC4 is the predominant clonal complex in CSF isolates among *Cronobacter* species.

Table 1.7: *C. sakazakii* strains of clinical importance commonly referred to in the literature.

Strain	Year	Clinical presentation	ST	Presence of ESBL	Reference
658[a]	2001	Meningitis	1	Unknown	1 & 4
2[b]	Unknown	Unknown	3	Unknown	1
695	1994	Fatal NEC II	4	Present	2
701	1994	Fatal NEC III	4	Unknown	2
767	1994	Fatal meningitis	4	Present	2 & 4
20	2004	Unknown	4	Unknown	2 & 3
1[c]	1980	Unknown	8	Unknown	3 & 4
696	1994	NECII	12	Unknown	2 & 3
680[d]	1977	Unknown	8	Uknown	4 & 5

ST: Sequence type.
NEC: Necrotising enterocolitis.
ESBL: Extended spectrum β-lactamase.
1: (Townsend *et al.* 2007b).
2: Caubilla-Barron *et al.* (2007).
3: Joseph and Forsythe (2011).
4: Kucerova *et al.* (2010).
5: Joseph *et al.* (2012b)
[a]: ATCC BAA-894
[b]: ATCC 12868
[c]: ATCC 29544 & NCTC 11467.
[d]: CDC 996-77.

1.9. Aims

The former discussion has shown that *C. sakazakii* can be isolated from different sources and that it is associated with severe and fatal cases of NEC and meningitis in neonates and infants. It is clear that the organism has a group of virulence traits that can influence its ability to cause deleterious clinical outcomes. It was reported that *C. sakazakii* was the dominant species from clinical sources across the *Cronobacter* genus (Joseph and Forsythe 2012c, Joseph *et al.* 2012d, Forsythe *et al.* 2014), and *C. sakazakii* CC4 was the most prevalent clonal complex of meningitis and CSF isolates (Joseph and Forsythe 2011, Joseph *et al.* 2012d, Forsythe *et al.* 2014). Although there have been huge advancements in the genome studies of the organism, the host response and its role in outcome remains unclear. It is hypothesised that the *C. sakazakii* is able to pass through the host intestinal barrier by altering the tight junctions to reach the bloodstream. Additionally, this pathogen might be capable of surviving within human phagocytes and of withstanding serum-mediated killing. Thus, it could cause bacteraemia and spread throughout the body reaching the BBB. It is also proposed that the organism can alter the tight junctions of the BBB and stimulate the host response resulting in the

release of inflammatory mediators. Such mediators might increase the permeability of the BBB thus allowing a subsequent migration of the infected phagocytes. In addition, it can trigger apoptosis of HBMEC and microglial cells that could affect the integrity of the BBB barrier.

Therefore, the initial aim of this project is to assess the virulence potential of *C. sakazakii* CC4 and non-CC4 clinical strains from different sources (e.g. CSF, blood, faeces) and clinical outcomes (e.g. meningitis, NEC, unknown). The first step to achieve that is studying the organism's ability to attach, invade, and translocate through a set of cell lines. The research also aims to examine the cytotoxicity of the bacterium and its ability to survive serum-mediated killing and phagocytosis. Following from this, the impact of *C. sakazakii* infection on human brain cells *in vitro* will be investigated. In addition, the host response to the infection will be analysed to clarify whether the severe outcomes are attributed to it or if they are entirely caused by the bacterium *per se*. This work will involve a range of phenotypic and molecular assays in order to provide accurate assessment and evaluation of the host response.

Chapter 2: Materials and Methods

The following chapter contains the common methods that were used in this project. Specific protocols will be detailed in the relevant chapter where appropriate.

2.1. Safety considerations

All experiments and protocols in this project were carried out in accordance to health and safety code of practice for microbiology level 2 containment laboratories at Nottingham Trent University. All protocols and materials' preparation were assessed and appropriate COSHH forms were completed. According to tissue culture laboratories health and safety regulations, Hepatitis B antibodies and vaccination were checked before conducting any tissue culture experiments. Waste was disposed according to the recommended procedures and material safety data sheets.

2.2. Sterilisation and aseptic techniques

All equipment, buffers, media, and solutions were sterilised by autoclave sterilisation at 121°C for 15 minutes, sprayed with 70% v/v ethanol, or filtered using 0.2 μm pore size filters (Fisher Scientific, UK) as appropriate. All protocols were carried out using aseptic techniques including use of a class II MSC hood to avoid contamination.

2.3. Reagents, buffers, and culture media

All reagents, media, and buffers used in this study are listed in Tables. 2.1-2 and were purchased either from Sigma Aldrich, UK or Fisher Scientific, UK unless otherwise specified.

Table.2.1: Reagents and buffers used in this project.

Reagents and buffers	Preparation method
Phosphate buffered saline (PBS)	2 tablet of PBS (Sigma Aldrich, UK) was dissolved in 400 ml of distilled water (dH$_2$O) and autoclaved.
Saline solution (0.85% v/v)	1 tablet of saline (Fisher Scientific, UK) was dissolved in 500 ml of dH$_2$O and autoclaved.
Triton X-100 (1% v/v)	1 ml of Triton X-100 (Fisher Scientific, UK) was added to 99 ml of dH$_2$O and autoclaved then stored at room temperature.
Glycerol (20% v/v)	2 ml of glycerol (Fisher Scientific, UK) was added to 80 ml of dH$_2$O, mixed, and autoclaved then stored at room temperature.
1X Tris-acetate-EDTA (TAE)	20 ml of 50X TAE buffer (Geneflow, UK) was mixed in 980 ml of dH$_2$O.
Iron III solution (1 mM FeCl$_3$–6H$_2$O, 10 mM HCl)	83 μl of concentrated HCl (12 M; Fisher Scientific, UK) were added into 100 ml of dH$_2$O to make 10 mM HCl. Then, 27 mg of FeCl$_3$ (Sigma Aldrich, UK) were dissolved in 1 L of dH2O. To Make 10 ml of mM iron III solution, 9 ml of 10 mM HCl were mixed with 1 ml of FeCl$_3$ solution.
3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; 5 mg/ml)	0.01 g of MTT (Sigma Aldrich, UK) was dissolved in 20 ml of PBS and then filtered through 0.2 μm pore size filter.
Giemsa stain (5% v/v)	10 ml of Giemsa stain (Life Technologies, UK) were diluted in 200 ml of sterile PBS at the time of the assay.

Table.2.2: Culture media used in this project.

Culture media	Preparation method
Tryptone soya agar (TSA)	40 g of TSA powder (Oxoid, UK) was dissolved in 1 L of dH$_2$O and then autoclaved before cooling to 50°C. The medium was then poured into Petri dishes, dried, and was stored at 4°C.
Tryptone Soya broth (TSB)	15 g of TSB powder (Oxoid, UK) was dissolved in 500 ml of dH$_2$O and then autoclaved before cooling to 60°C and was stored at room temperature.
Luria-Bertani broth (LB)	25 g of LB powder (Sigma Aldrich, UK) was dissolved in 1 L of dH$_2$O and then autoclaved before cooling to 60°C and was stored at room temperature.

2.4. Bacterial strains

Thirty-four *C. sakazakii* strains were used in this research. These isolates were selected from Nottingham Trent University culture collection including the control strains *S*. Enteritidis NTU 358 (+ve), *Cit. koseri* NTU 48 (+ve), and *E. coli* K12 NTU 1230 (-ve; Table.2.3). The strains were chosen according to their sequence type, source, and clinical outcomes. This included a group of well-characterised strains from the 1994 French outbreak (Caubilla-Barron *et al.* 2007). Strains were stored in TSB (Oxoid, UK) containing 20% v/v glycerol at -20°C and -80°C. They were grown on TSA (Oxoid, UK) under aerobic conditions at 37°C for 18 hours. Up-to-date information and details about the isolates can be found at http://www.pubmlst.org/cronobacter.

Table.2.3: *C. sakazakii* strains used in this research.

Strain	ST	Source	Clinical presentation	Country	Genome sequenced
6	4 [b]	Clinical - Unknown	Unknown	Canada	Yes (1)
20	4 [b]	Clinical - Unknown	Unknown	Czech Republic	Yes (1)
553	4 [b]	Clinical - Unknown	Unknown	Netherlands	Yes (1)
557	4 [b]	Clinical - Unknown	Unknown	Netherlands	Yes (1)
558	4 [b]	Clinical - Unknown	Unknown	Netherlands	Yes (1)
695[a]	4 [b]	Clinical - Trachea	Fatal NEC II	France	Yes (1)
721	4 [b]	Clinical - CSF	Unknown	USA	Yes (1)
730[a]	4 [b]	Clinical - Unknown	NEC I	France	Yes (1)
767[a]	4 [b]	Clinical - Trachea	Fatal meningitis	France	Yes (1)
1219	4 [b]	Clinical - CSF	Fatal meningitis	USA	Yes (1)
1220	4 [b]	Clinical - CSF	Brain abscess, not fatal	USA	Yes (1)
1221	4 [b]	Clinical - CSF	Meningitis	USA	Yes (1)
1222	4 [b]	Clinical - Blood	Fever	USA	No
1223	4 [b]	Clinical - Blood	Unknown	USA	No
1224	4 [b]	Clinical - Blood	Fever	USA	No
1225	4 [b]	Clinical - Blood	Fatal meningitis	USA	Yes (1)
1231	4 [b]	Clinical - Faeces	Meningitis	New Zealand	Yes (1)
1240	4 [b]	Clinical - CSF	Fatal meningitis	USA	Yes (1)
1242	4 [b]	Clinical - Brain	Fatal meningitis	USA	No
1465	4 [b]	Infant formula	Unknown	Saudi Arabia	No
1587	109[b]	Clinical - CSF	Brain damage	Israel	Yes (1)
4	15[b]	Clinical - Unknown	Unknown	Canada	Yes (1)
1249	31	Clinical - Unknown	Fatal infant isolate	UK	Yes (1)
12	1	Clinical - Faeces	Unknown	Czech Republic	No
555	1	Clinical - Unknown	Unknown	Netherlands	No
658[c]	1	Non-infant formula	Meningitis	USA	Yes (3)
1019[d]	1	Clinical - CSF	Meningitis	USA	No
1241	1	Clinical - Blood	Unknown	USA	No
1	8	Clinical - Throat	Unknown	USA	Yes (1)
5	8	Clinical - Unknown	Unknown	Canada	Yes (1)
680	8	Clinical - CSF	Unknown	USA	Yes (2)
520	12	Clinical - Unknown	Unknown	Czech Republic	Yes (1)
696[a]	12	Clinical - Faeces	NEC II	France	Yes (2)
580	18	Clinical - Unknown	Unknown	UK	No

ST: sequence type.
NEC: necrotising enterocolitis.
CSF: cerebrospinal fluid.
[a]**:** French outbreak strains (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2008).
[b]**:** Part of clonal complex 4, which contains STs 4, 15, 97, 107, 108 and 109.(Forsythe *et al.* 2014).
[c]**:** BAA-894 the first *C. sakazakii* strain sequenced (Kucerova *et al.* 2010).
[d]: Alternative culture collection code HPB-3290.
(1): Exeter, unpublished.
(2): Life Technologies (Joseph *et al.* 2012b)
(3): Washington (Kucerova et al. 2010).

For the PhD stage, thirteen *C. sakazakii* strains (ST4 and non-ST4) were selected based on several criteria obtained from the MPhil stage such as the site of isolation and the nature of infection, the high invasiveness and translocation, macrophage persistence, and iNOS induction. More information about these strains is listed in Table.2.4.

Table.2.4: *C. sakazakii* strains selected for detailed analysis.

Strain	ST	Source	Clinical presentation	Country	Genome Sequenced
6	4	Clinical	Unknown	Canada	Yes (1)
20	4	Clinical - Faeces	Unknown	Czech Republic	Yes (1)
558	4	Clinical	Unknown	Netherlands	Yes (1)
695[a]	4	Clinical - Trachea	Fatal NEC II	France	Yes (1)
767[a]	4	Clinical - Trachea	Fatal meningitis	France	Yes (1)
1221	4	Clinical - CSF	Meningitis	USA	Yes (1)
1240	4	Clinical - CSF	Fatal meningitis	USA	Yes (1)
1242	4	Clinical - Brain	Fatal meningitis	USA	No
1587	109[b]	Clinical - CSF	Brain damage	Israel	Yes (1)
1249	31	Clinical	Fatal infant isolate	UK	Yes (1)
658[c]	1	Non-infant formula	Meningitis	USA	Yes (3)
680	8	Clinical - CSF	Unknown	USA	Yes (2)
696[a]	12	Clinical - Faeces	NEC II	France	Yes (2)

ST: sequence type.
NEC: necrotising enterocolitis.
CSF: cerebrospinal fluid.
[a]: French outbreak strains (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2008).
[b]: New CSF isolate that was received during the project period and added to the strains in the final stage of this study. Part of clonal complex 4, which contains STs 4, 15, 97, 107, and 108 (Joseph *et al.* 2012d).
[c]: BAA-894 the first *C. sakazakii* strain sequenced (Kucerova *et al.* 2010).
(1): Exeter, unpublished.
(2): Life Technologies (Joseph *et al.* 2012b).
(3): Washington (Kucerova *et al.* 2010).

2.5. *C. sakazakii* virulence

2.5.1 Motility test

Four grams of microbiology nutrient agar No.2 (Fluka, UK) and thirty grams of TSB (Oxoid, UK) in 1 L of water mixture were prepared and autoclaved to make soft agar plates. Strains were grown in LB broth and incubated at 37°C for 18 hours with shaking (180rpm). The samples were then diluted to 10^{-4} in PBS before inoculating by stabbing into the soft agar plate at 3 µl of bacterial suspension. The test was undertaken twice with three inoculations each. The motility was determined by measuring the zone of growth around the inoculation spot after 18 hours growth at 37°C.

2.5.2. Iron siderophore detection

Chrome Azurol S (CAS) agar diffusion (CASAD) assay was used to detect iron siderophore production. The experiment was prepared as described previously (Shin *et al.* 2001). Initially, 60.5 mg of CAS (Sigma Aldrich, UK) was dissolved in 50 ml deionised water and then mixed with 10 ml of iron III solution (Table.2.1). The mixture was added with stirring into 72.9

mg of Hexadecyltrimethylammonium (HDTMA; Sigma Aldrich, UK) in 40 ml of water. This dark solution was autoclaved and mixed with a second autoclaved solution containing 900 ml water, 15 g agarose (Sigma Aldrich, UK), 30.24 g PIPES (Sigma Aldrich, UK), and 12 g of NaOH solution (50% w/v in water). The mixture was poured in petri dishes and left to solidify. Wells were cut in the plates with a 5 mm diameter gel plug cutter and stored at 4°C.

Bacterial strains were grown on TSA at 37°C for 18 hours incubation. Five colonies were inoculated in 10 ml of LB broth containing 200 µM 2, 2'-Dipyridyl (Sigma Aldrich, UK) and incubated with shaking at 200 rpm at 37°C for 18 hours. The tubes were then centrifuged at 5000 rpm (Megafuge 16R-Thermo Scientific, UK) for 10 minutes. Cell free supernatant of 70 µl of each sample was added into the 5 mm holes that were cut in the agar and plates incubated for 4-8 hours at 37°C. a volume of 70 µl of PBS was added into some wells as a negative control.

2.5.3. *C. sakazakii* sensitivity to human serum

The sensitivity of *C. sakazakii* strains to active human serum was conducted as described previously by Hughes *et al.* (1982) with slight modification. Bacterial cultures were grown for 18 hours in LB at 37°C with shaking at 200 rpm then centrifuged for 10 minutes at 1300 rpm (Mikro 200-Hettik). The pellet was then re-suspended to 10^6 cfu/ml in 5 ml of phosphate buffered saline (PBS; Sigma Aldrich, UK). A volume of 0.5 ml of the suspension was added into 1.5 ml of undiluted active human serum (Sigma Aldrich, UK). The samples were loaded into a 24-well plate and incubated at 37°C with shaking (200 rpm). Viable counts were obtained at 4 different time points (0, 1, 2, and 3 hours) after serial dilution in PBS and plating on TSA for 18 hours at 37°C. Each strain was tested in triplicate, and the mean results were stated as percent survival of inoculum.

$$\% \text{ Survival in serum} = \frac{\text{The total number of surviving bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

2.5.4. Comparative genomic analysis for *C. sakazakii* virulence genes

A group of genes of interest were chosen according to the literature and Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Ogata *et al.* 1999, Franco *et al.* 2011a, Franco *et al.* 2011b, Grim *et al.* 2012). These genes were linked to important virulence traits such as serum resistance and iron acquisition.

Table.2.5: Putative virulence genes that were investigated in this project.

Gene	Description	Function	GenBank accession number	Reference
fliA	Flagellar biosynthesis sigma factor.	Motility-flagella	ESA_01300[A]	1, 2
fliC	Flagellin.	Motility-flagella	ESA_01288[A]	1, 2
fliD	Flagellar hook-associated protein.	Motility-flagella	ESA_01287[A]	1, 2
fliE	Flagellar hook-basal body complex protein.	Motility-flagella	ESA_01261[A]	1, 2
fliF	Flagellar motor switch ring protein.	Motility-flagella	ESA_01260[A]	1, 2
fliG	Flagellar motor switch protein G.	Motility-flagella	ESA_01259[A]	1, 2
fliH	Flagellar assembly protein H.	Motility-flagella	ESA_01258[A]	1, 2
fliI	Flagellum-specific ATP synthase.	Motility-flagella	ESA_01257[A]	1, 2
fliJ	Flagellar biosynthesis chaperone.	Motility-flagella	ESA_01256[A]	1, 2
fliK	Flagellar hook-length control protein.	Motility-flagella	ESA_01255[A]	1, 2
fliL	Flagellar basal body-associated protein.	Motility-flagella	ESA_01254[A]	1, 2
fliM	Flagellar motor switch protein.	Motility-flagella	ESA_01253[A]	1, 2
fliN	Flagellar motor switch protein.	Motility-flagella	ESA_01252[A]	1, 2
fliO	Flagellar biosynthesis protein.	Motility-flagella	ESA_01251[A]	1, 2
fliP	Flagellar biosynthesis protein.	Motility-flagella	ESA_01250[A]	1, 2
fliQ	Flagellar biosynthesis protein.	Motility-flagella	ESA_01249[A]	1, 2
fliR	Flagellar biosynthesis protein.	Motility-flagella	ESA_01248[A]	1, 2
fliS	Flagellar protein.	Motility-flagella	ESA_01286[A]	1, 2
fliT	Flagellar biosynthesis protein.	Motility-flagella	ESA_01285[A]	1, 2
fliY	Cysteine transporter subunit.	Motility-flagella	ESA_01302[A]	1, 2
fliZ	Flagella biosynthesis protein.	Motility-flagella	ESA_01301[A]	1, 2
eitA	ABC transporter, periplasmic substrate-binding component.	Iron acquisition	ESA_pESA3p05518[A]	3, 4
iucC	Siderophore synthetase superfamily, group C.	Iron acquisition	ESA_pESA3p05549[A]	3, 4
viuB	Iron-chelator utilisation protein, siderophore-encoding gene.	Iron acquisition	ESA_pESA3p05545[A]	4, 5
cpa	Outer membrane protease responsible for serum resistance	Serum resistance	ESA_pESA3p05434[A]	6
rcsA	Colanic acid capsular biosynthesis activation protein A.	Colanic acid production	ES15_1478[B]	7
apaH	Diadenosine tetraphosphatase.	Invasion	ECs0054[C]	8
ompA	Outer membrane protein A.	Invasion	ESA_02391[A]	1, 2
ompX	Outer membrane protein X.	BMEC invasion	ESA_02526[A]	1, 2
ygdP	Dinucleoside polyphosphate hydrolase.	BMEC invasion	APECO1_3675[D]	9
ppk1	Polyphosphate kinase.	BMEC invasion	APECO1_4068[D]	10
phoP	Virulence transcriptional regulator PhoP.	Phagocytosis survival	STM1231[E]	11
phoQ	Virulence sensor histidine kinase PhoQ.	Phagocytosis survival	STM1230[E]	11
pmrA	DNA-binding transcriptional regulator BasR.	Phagocytosis survival	ESA_03574[A]	11
pmrB	Sensor protein BasS/PmrB.	Phagocytosis survival	ESA_03575[A]	11
pmrE	UDP-glucose dehydrogenase.	Phagocytosis survival	ESA_01534[A]	11
mgtB	Magnesium-transporting ATPase.	Phagocytosis survival	STM3763[E]	12
gsrA	Serine endoprotease.	Phagocytosis survival	YPO3382[F]	13
sodA	Superoxide dismutase.	Phagocytosis survival	ESA_03843[A]	1, 14

[A]: *Cronobacter sakazakii* strain 658, the first *Cronobacter* sequenced strain (Kucerova *et al.* 2010). [B]: *Cronobacter sakazakii* strain ES15 (Shin *et al.* 2012). [C]: *E. coli* O157:H1. [D]: *E. coli* APECO1. [E]: *S. enterica serovar* Typhimurium LT2. [F]: *Y. pestis* CO92.
1: (Kucerova *et al.* 2010). **2:** Joseph *et al.* (2012b). **3:** Franco *et al.* (2011a). **4:** Grim *et al.* (2012). **5:** Bogard and Oliver (2007). **6:** Franco *et al.* (2011b). **7:** Gottesman *et al.* (1985). **8:** Ismail *et al.* (2003). **9:** Badger *et al.* (2000). **10:** Peng *et al.* (2012). **11:** Ernst *et al.* (1999). **12:** Blanc- Potard and Groisman (1997). **13:** Yamamoto *et al.* (1996). **14:** Townsend *et al.* (2007b).

2.5.4.1. Screening of *C. sakazakii* plasmid associated serum resistance and iron acquisition genes

Plasmid extraction, primer design, and the PCRs were all conducted according to Franco *et al.* (2011a) with some modifications.

2.5.4.1.1. Plasmid DNA extraction

Plasmid extraction was carried out according to the manufacturer's instructions using QIAprep Spin Miniprep Kit (Qiagen, UK). Bacterial strains were grown on TSA (Oxoid, UK) from frozen cultures at -20°C. A single colony from each strain was inoculated in 3 ml of LB broth and incubated at 37°C for 18 hours with shaking (180 rpm). A volume of 2 ml of overnight (18 hours) culture was centrifuged at 8000 rpm (Mikro 200-Hettich) for 3 minutes at room temperature, and then the pellet was re-suspended in 250 µl of Buffer P1 and transferred to a micro-centrifuge tube. Next, 250 µl of Buffer P2 were added and mixed 4-6 times by inverting, until the solution became clear. Afterwards, 350 µl of Buffer N3 were pipetted into the tube and mixed immediately 4-6 times by inverting. The tube then was centrifuged for 10 minutes at 13000 rpm in a table-top micro-centrifuge (Mikro 200-Hettich). The supernatant was then decanted to a QIAprep spin column before centrifugation at 13000 rpm (Mikro 200-Hettich) for 60 seconds. Next, the column was washed by 500 µl of Buffer PB and centrifuged at 13000 rpm (Mikro 200-Hettich) for 60 seconds. Following centrifugation, the column was washed by adding 750 µl of Buffer PE and centrifuged for 60 seconds at 13000 rpm (Mikro 200-Hettich) before discarding the flow-through. The column was centrifuged again at 13000 rpm (Mikro 200-Hettich) for 60 seconds to remove the residual wash buffer. The column was then transferred to a clean 1.5 ml collection tube. Finally, 50 µl of Buffer EB were added to the centre of the QIAprep spin column and incubated for 60 seconds to elute DNA before centrifugation at 13000 rpm (Mikro 200- Hettich) for 60 seconds.

2.5.4.1.2. Polymerase chain reaction (PCR) protocol

PCR primers were designed to target *cpa, iucC*, and *eitA* loci on the large *C. sakazakii* plasmid pESA3. GoTaq® DNA Polymerase kit (Promega, UK) was used for all PCR reactions' preparation (Table.2.6.). Information

about PCR reactions is listed in Table.2.7. The PCR products were analysed using agarose gel electrophoresis.

Table.2.6: PCR master mix components and volumes.

Constituent	Volume	Final concentration
GoTaq® reaction buffer, 5X	5 µl	1X
MgCl$_2$	2 µl	1.5 mM
dNTP mix	1 µl	0.2 mM each dNTP
Upstream primer*	2.5 µl	10 µM
Downstream primer*	2.5 µl	10 µM
Taq polymerase	1 µl	1.25 Unit Taq
DNA template	1 µl	~ 10 ng
Nuclease-Free Water	10 µl	NA

NA: not applicable
*: Purchased from Eurofin, UK.

Table.2.7: PCR primers and reactions used in this study (Franco *et al.* 2011a).

Target	Function	Primer	Sequence (5'-3')	Amplicon (bp)	Annealing/ Extension Cycle parameters[1]
cpa	Serum resistance	cpafw cparv	GACAACCCTGAGTTCTGGTAAC ATGCGTATTTCTGCTGGTAA	360	56°C 30s; 72°C 30s
iucC	Iron acquisition	IucCF IucCR	TGCAGTGCCTGATGTCAGGCCAT ACGCCAAACATCTCCTGATAGCG	660	58°C 30s; 72°C 30s
eitA	Iron acquisition	EitAF1 EitAR1	CCTTTTTCACGGCGTCGAGCTG TCTCTTCTGGTTCTCCAGCGCG	280	60°C 30s; 72°C 30s

[1] All reactions started with 3 minutes at 94°C followed by 25 cycles including denaturation step at 94°C for 30 seconds.
s: seconds.

2.5.4.1.3. DNA separation using agarose gel electrophoresis

All amplified PCR products were visualised using a 1% w/v agarose gel. To prepare 1% (w/v) agarose gel, 0.5 g of agarose (Fisher Scientific, UK) were mixed with 50 ml of 1X TAE buffer (Geneflow, UK; Table.2.1). The mixture then was heated in the microwave oven until the agarose was completely dissolved. Next, 5 µl (0.1 µl/ml v/v) of SYBER® Safe DNA gel stain (Life Technologies, UK) was added into the mixture and mixed well before it was poured into the gel tray and left to set.

The gel was then submerged in a horizontal running tank, Mini-Sub® Cell GT (Bio-Rad Laboratories Ltd., UK), containing 1X TAE buffer. Afterwards, 5 µl of each PCR product was loaded into each well, and 5 µl 1 kb DNA ladder (Promega, UK) was loaded to determine the size of the DNA fragments. The gel was then run at 100V for 40 minutes. Finally, using InGenius® gel documentation system (Syngene; UK), the gel was examined under ultraviolet (UV) to view the DNA bands.

2.5.4.2. Genome comparisons for the presence/absence of possible *C. sakazakii* virulence genes

To investigate the presence and the absence of the key genes, genome comparisons were applied to find out the unique regions of interest that may contribute to the virulence of the organism. This comparative analysis was done using Artemis genome browser to investigate the presence/absence of desired genes and Artemis comparison tool (WebACT) for genome pairwise alignments (Rutherford *et al.* 2000, Carver *et al.* 2005). Moreover, BLAST genome search was applied to strains using the sequence of each gene to find out if they are present or not. The BLAST search was at http://www.pubmlst.org/cronobacter.

2.5.5. Tissue culture-based virulence studies

2.5.5.1. Mammalian cell lines

Cell lines used in this project (Table.2.8) were preserved in liquid nitrogen until they were required. To grow the cell line, a vial of the appropriate cell line was taken from liquid nitrogen and kept in ice. A volume of 20 ml of pre-warmed tissue culture growth medium (see Table.2.9) was added to 75-cm^3 tissue culture flask. The cell line vial then was thawed quickly and pipetted into the culture flask before incubation at 37°C in 5% CO_2 for up to 24 hours. Afterwards, the growth medium was replaced by 20 ml of fresh medium to remove the residues of cell line preservatives. The flask was incubated for 48-72 hours to achieve a confluent monolayer of cells. The cells then were detached using 5 ml of TrypLe™ express (Life Technologies, UK) and centrifuged for 3 minutes at 1500 rpm using LMC-3000 centrifuge (Grant-bio, UK). The supernatant was discarded and the pellet re-suspended in growth medium before transferring into a new flask

containing 20 ml of fresh growth medium. The cell lines were maintained and split two times a week to keep continuity of growth.

Table.2.8: Cell lines used in this project.

Cell line	Passages	Reference No.	Source
Colorectal adenocarcinoma epithelial cells (Caco-2)	17-45	ECACC #86010202	European Collection of Cell Cultures
Human brain microvascular endothelial cells (HBMEC)	2-25	#P10354	Inooprot, Spain
Macrophage cell line (U937)	12-28	ATCC#CRL-1593.2	American Type Culture Collection
Human microglial cell line (HMGC)	3-17	#HMG030	Inooprot, Spain
Rat brain capillary endothelial cell line (rBCEC4)	21-36	-	I. E. Blasig*

*: Forschungsinstitut für Molekulare Pharmakologie – Berlin, Germany.

Table.2.9: Tissue culture media used in this project.

Cell line	Growth medium	Infection medium
Caco-2	Minimum Essential Medium (MEM) supplied with 10% (v/v) foetal calf serum (FCS), 1% (v/v) non-essential amino acid (NEAA), and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK).	Minimum Essential Medium (MEM) supplied with 10% (v/v) FCS, and 1% (v/v) non- NEAA (Sigma Aldrich, UK).
HBMEC	Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK)	Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS (Sigma Aldrich, UK)
rBCEC4	Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK)	Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FCS (Sigma Aldrich, UK)
U937	RPMI medium containing 10% (v/v) FCS, 1% (v/v) NEAA, and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK)	RPMI medium containing 10% (v/v) FCS and 1% (v/v) NEAA (Sigma Aldrich, UK).
HMGC	Basal medium containing 10% (v/v) FCS, 10% (v/v) microglial growth supplement, and 1% (v/v) penicillin-streptomycin (Innoprot, Spain).	Basal medium containing 10% v/v FCS, and 1% (v/v) microglia growth supplement (MCGS) (Innoprot, Spain).

2.5.5.2. Preparing bacterial inocula

A single colony of each test strain was inoculated into 5 ml of LB (Oxoid, UK) and grown aerobically at 37°C for 18 hours prior to infection with shaking at 200 rpm. On the day of the assay, 120 μl of overnight (18 hours) cultures were added into 5 ml of infection medium (Table.2.9) before being incubated at 37°C with shaking at 200 rpm for 1.5 hours to achieve an optical density (OD) of 0.3 - 0.5 at 600_{nm} using the spectrophotometer (JENWAH, UK). The bacterial suspension was then diluted in infection medium to obtain 4×10^6 cfu/ml, which is multiplicity of infection of 100 (MOI 100) for Caco-2, HBMEC, and rBCEC4 cell lines. For U937 and HMGC cell lines, bacterial suspension was diluted to 4×10^5 cfu/ml (MOI 10).

2.5.5.3. Bacterial cytotoxic activity to human cell lines (MTT)

This assay is based on the reduction of MTT (Sigma Aldrich, UK) by viable cells to its insoluble form formazan, which has a purple colour. This experiment was applied as described previously (Kielian *et al.* 2004, Krzymińska *et al.* 2009, Liu and Kielian 2009, Travan *et al.* 2009) with some modifications. Human cells, Caco-2, human brain microvascular endothelial cells (HBMEC), and human microglial cells (HMGC), were grown in 24-well plates at 4×10^4 cell/well. The plates were then incubated for 48 hours at 37°C in 5% CO_2. Bacterial suspensions were prepared by growing bacteria for 18 hours prior to infection. These were then added to the wells at 4×10^6 cfu/well MOI 100 and 4×10^5 cfu/well MOI 10 for HMGC. The plates were then incubated in 5% CO_2 at 37°C for 1 and 3 hours. The wells were then washed three times using PBS before adding 0.5 ml fresh tissue culture infection medium. A volume of 50 μl of MTT at a concentration of 5 mg/ml (w/v; Table.2.1) was added to each well. The plates were then incubated in 5% CO_2 for 1 and 3 hours at 37°C. Next, the medium containing MTT was removed and formazan was solubilised in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). The plates were then shaken for 10 minutes before measuring the absorbance at 600_{nm} using a plate reader (BioTek, UK). The negative control for the assay consisted of uninfected cells.

2.5.5.4. *C. sakazakii* attachment and invasion assays

The experiment was carried out to assess the ability of the isolates to attach and invade human cells. This assessment was determined *in vitro* by applying the gentamicin protection assay using Human colonic carcinoma epithelial cells (Caco-2) passages 17 to 45 acquired from the European Collection of Cell Cultures (ECACC #86010202), human brain microvascular endothelial cells passages 2 to 25 (HBMEC; ref. #HMG030 Inooprot, Spain) and rat brain capillary endothelial cell line (rBCEC4) passages 21 to 36, which was obtained from I. E. Blasig (Forschungsinstitut für Molekulare Pharmakologie – Berlin, Germany; Table.2.8). The tissue culture media used in this assay are listed in Table.2.9. All experiments used consistent conditions of time, temperature, cell line passage, mammalian cell concentration, and bacterial suspension densities. *Salmonella* Enteritidis strain NTU 358 was used as positive control for the Caco-2 cell line, while *Citrobacter koseri* strain NTU 48 was the positive control for HBMEC and rBCEC4 cell lines. *Escherichia coli* K12 strain NTU 1230 was the negative control for all cell lines. Control strains were from the NTU strain collection.

2.5.5.4.1. Bacterial attachment to mammalian cells

This experiment was as described previously by Townsend *et al.* (2008) with slight modifications. Mammalian cells were grown as mentioned previously in Section 2.5.5.1. and then seeded in 24-well plates (Sarstedt, Germany) at 4×10^4 cell/well in growth medium (Table.2.9) for 48 hours in 5% CO_2 at 37°C to achieve a confluent monolayer. *C. sakazakii* strains were grown as described in Section 2.5.5.2. The suspensions were then added to the wells at 4×10^6 cfu/well MOI 100, and incubated in 5% CO_2 at 37°C for 2 hours. The wells were then washed three times using PBS (Sigma Aldrich, UK), and lysed with 1% (v/v) Triton X-100 (Fisher Scientific, UK; Table.2.1). The lysates were serially diluted in PBS and then plated on TSA for 18 hours at 37°C to determine the viable count. Data are presented as the percentage efficiency of attachment.

$$\% \text{ efficiency of attachment} = \frac{\text{The total number of attached bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

2.5.5.4.2. Bacterial invasion of mammalian cells

The method was as per bacterial attachment above. However, following the washing three times by PBS, 0.5 ml of infection medium (Table.2.9) supplied with 125 µg/ml of gentamicin, which is the lethal concentration for *Cronobacter* (Sigma Aldrich, UK) was added and incubated in 5% CO_2 at 37°C for 1 hour. The wells were then washed once with PBS (Sigma Aldrich, UK) before lysing by 1% (v/v) Triton X-100 (Fisher Scientific, UK; Table.2.1), and plated on TSA at 37°C for 18 hours incubation after serial dilution in PBS to obtain viable count. Data are presented as the percentage efficiency of invasion.

$$\% \text{ efficiency of invasion} = \frac{\text{The total number of invaded bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

2.5.5.5. Translocation assay

2.5.5.5.1. Translocation assay using Caco-2 cell line

Translocation assay was performed as previously described (Burns *et al.* 2001, Giri *et al.* 2011). However, 0.8 ml of growth medium (Table.2.9) was added to the basolateral chamber of Millicell-24 cell culture plate (Millipore, UK). Caco-2 cells, at 4×10^4 cell/well in 0.4 ml/well of growth medium, were seeded onto a 3 µm pore polycarbonate transwell membrane in the apical chamber of the tissue culture plate and incubated in 5% CO_2 at 37°C. The medium in the apical and the basolateral chambers was changed every 3 days. Millicell ERS-2 Volt-Ohm Meter (Millipore, UK) was used to measure the transepithelial electrical resistance (TEER). According to the literature, the Caco-2 cell line required up to 21 days to form intact polarised monolayers with TEER 300-850 Ωcm^{-2} (Finlay and Falkow 1990, Burns *et al.* 2001, Giri *et al.* 2011).

On the day of the assay, the medium in the basolateral chamber was replaced with infection medium (Table.2.9). The medium in the apical chamber was removed, and the membrane was washed two times using 0.4 ml of PBS (Sigma Aldrich, UK). Bacterial suspensions was prepared as described previously in Section 2.5.5.2, then 0.4 ml of each suspension containing 4×10^6 cfu (MOI 100) or medium alone as control was added to the apical chamber. At each time point of incubation (1, 3, and 5 hours),

the basolateral chamber was sampled for viable count after serial dilution in PBS and inoculated on TSA. After sampling the medium in the basolateral chamber was replaced by fresh medium.

$$\% \text{ efficiency of translocation} = \frac{\text{The total number of translocated bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

The transepithelial electrical resistance (TEER) was measured at four different time points (0, 1, 3, 5 hours) using a Millicell ERS-2 Volt-Ohm Meter (Millipore, UK) (Fig.2.1). Data are presented as the percentage efficiency of translocation.

2.5.5.5.2. Translocation assay using HBMEC cell line

This assay was carried out using the protocol described previously (Nizet *et al.* 1997, Badger *et al.* 1999, Giri *et al.* 2011). The basolateral chambers of the 24-well plate were filled with 0.510 ml/well of growth medium (Table.2.9). Cells, with a concentration of 4×10^4 cell/well in 0.375 ml growth medium (Table.2.9), were seeded onto the apical part of collagen-coated polytetrafluoroethylene (PTFE) membrane with a pore size of 0.4 μm (Transwell-COL; Corning, USA), and incubated in 5% CO_2 at 37°C. The medium in the apical chamber was changed every 3 days. The transendothelial electrical resistance (TEER) was measured using Millicell ERS-2 Volt-Ohm Meter (Millipore, UK). According to electrical resistance measurements, the HBMEC cell line required 5 to 8 days to form intact polarised monolayers with TEER 300-600 Ωcm^{-2} (Kim 2003, Giri *et al.* 2011).

Prior to infecting the cell line, the filter was washed twice with 0.375 ml/well PBS, and the medium in the basolateral part was replaced by infection medium (Table.2.9). The cell line was infected with 0.375 ml per well bacterial suspension prepared as previously mentioned in Section 2.5.5.2. with MOI of 100. The basolateral chamber was sampled and serially diluted in PBS at three time points of incubation (1, 3, and 5 hours) and then plated for viable count on TSA at 37°C before replacing with fresh infection medium. Data are presented as the percentage efficiency of translocation.

$$\% \text{ efficiency of translocation} = \frac{\text{The total number of translocated bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

The electrical resistance of the monolayers was also measured at 0, 1, 3, and 5 hours.
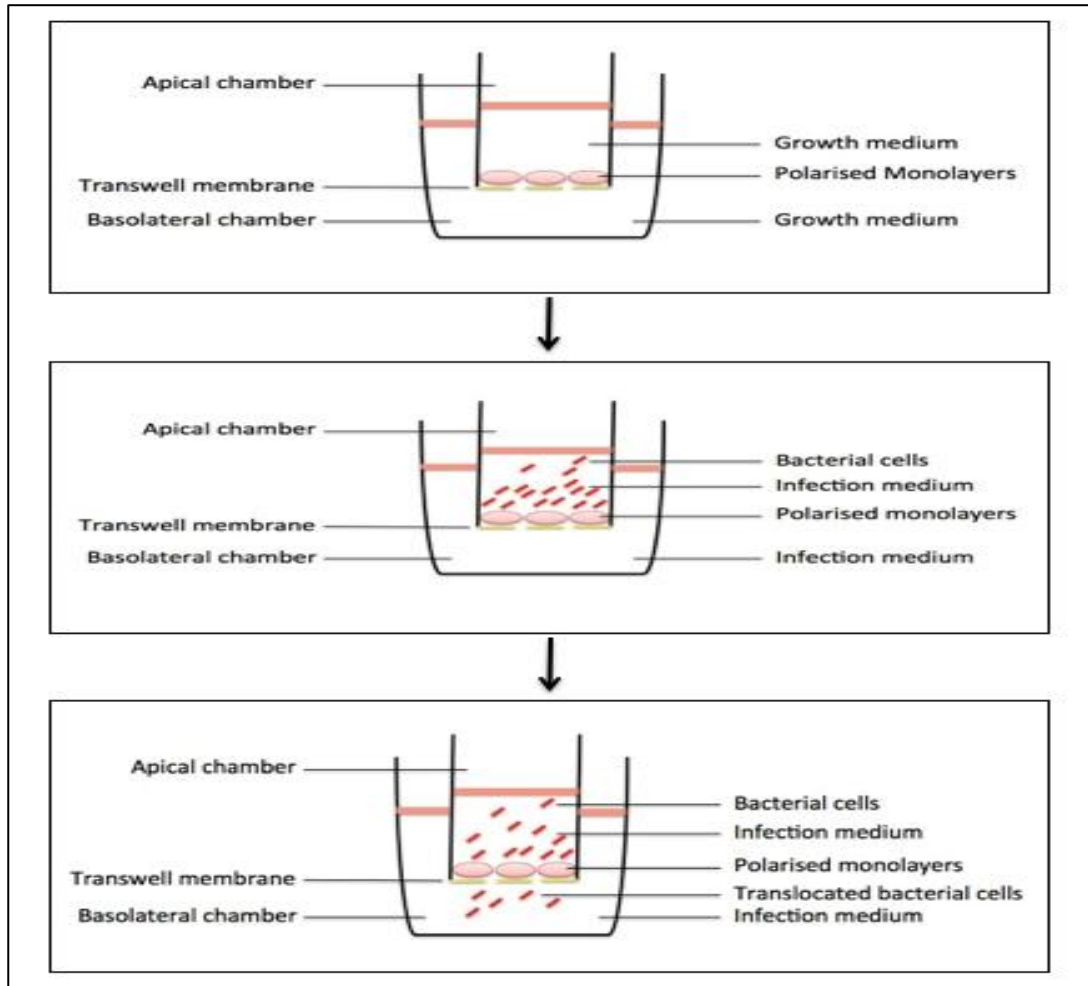
Fig.2.1 Steps of translocation assay.

2.5.5.6. Uptake and survival assays inside phagocytic cells

Uptake and survival assays were applied to investigate the ability of *C. sakazakii* strains to survive after being phagocytised by human macrophages and microglial cells. Macrophage cell line (U937) passage 12 was obtained from American Type Culture Collection (ATCC; #CRL-1593.2), and human microglial cell line passage 3 was obtained from Innoprot Technologies (Ref.# P10354). The media used in these experiments are listed in Table.2.9.

2.5.5.6.1. *C. sakazakii* persistence in human macrophages

As previously described by Townsend *et al.* (2007) with slight modifications, macrophages were grown as described in Section 2.5.5.1. and then treated with growth medium (Table.2.9) containing 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (Sigma Aldrich, UK) for maturation. Before seeding the 24-well plates, phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, UK) at a concentration of 0.1 µg/ml was added to cell suspensions to promote cell adhesion. The cells were then plated into 24-well plates at $4x10^4$ cell/well and incubated in 5% CO_2 at 37°C for 72 hours to produce confluent monolayers.

Macrophages were infected with overnight (18 hours) bacterial suspensions prepared as described in Section 2.5.5.2, however the concentration of bacterial cells was $4x10^5$ cfu/ml (MOI 10). The plates were then incubated 1 hour in 5% CO_2 at 37°C. After incubation, the medium was aspirated and replaced with infection medium (Table.2.9) containing 125 µg/ml of gentamicin, which is the lethal concentration for *Cronobacter*, and incubated in 5% CO_2 at 37°C for 1 hour. Four plates were then washed 3 times with PBS before addition of infection medium (Table.2.9) containing 50 µg/ml of gentamicin followed by further incubation (6 h, 24 h, 48 h, and 72 h). After each time point of incubation, the plates were washed twice with PBS before cells were lysed by addition of 0.120 ml of 1% (v/v) Triton X-100, and then serially diluted in PBS before plating on TSA to enumerate the intracellular bacteria at different time points (uptake, 6 h, 24 h, 48 h, and 72 h). Data are displayed as percentage of uptake and persistence at each time point.

$$\% \textit{ uptake and persistence} = \frac{\text{The total number of up taken/persisted bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \times 100$$

2.5.5.6.2. *C. sakazakii* persistence in human microglial cells

As previously described by Liu and Kielian (2009) with slight modifications, microglial cells were grown as stated in Section 2.5.5.1. using basal medium (Table.2.9) for three days in 75 cm^3 tissue culture flask. The cells then were seeded into four 24-well plates at $4x10^4$ cell/well and incubated in 5% CO_2 at 37°C for 48 hours to achieve

confluency. Next, the cells were infected with overnight (18 hours) bacterial suspensions prepared as explained in Section 2.5.5.2, however the bacterial cells were added at MOI of 10. Afterwards, the plates were incubated for 1 hour in 5% CO_2 at 37°C. The medium was then aspirated and replaced by infection medium (Table.2.9) containing 125 µg/ml of gentamicin, which is the lethal concentration for *Cronobacter*, and incubated in 5% CO_2 at 37°C for 1 hour after washing 3 times by PBS. Three plates were then washed 3 times with PBS and supplied with infection medium (Table.2.9) containing 50 µg/ml of gentamicin for further incubation (24 h, 48 h, and 72 h). At the end of each time point of incubation, the cells were washed twice with PBS before cells were lysed by addition of 0.120 ml of 1% (v/v) Triton X-100, and plated on TSA after being serially diluted in PBS to obtain the intracellular bacteria at different time points. Data are displayed as percentage of uptake and persistence at each time point.

$$\% \ uptake \ and \ persistence = \frac{\text{The total number of up taken/persisted bacterial cells (cfu/ml)}}{\text{The total number of bacterial cells in inoculum (cfu/ml)}} \ X \ 100$$

2.5.6. Visualisation of bacterial interaction with host cells

2.5.6.1. Adhesion assay

This assay was as described previously by Mange *et al.* (2006) and Wieler *et al.* (2011) with slight modifications. Caco-2 and HBMEC cells were seeded at $4x10^4$ cell/well in 6-well plates containing glass cover slips sterilised by heat, which were used to aid visualisation, and incubated at 37°C in 5% CO_2 for 48 hours to achieve confluency before infection. The test strains were prepared as described previously in Section 2.5.5.2. The cells then were infected with the bacterial suspension at $4x10^6$ cfu/well (MOI 100) and incubated for 2 hours at 37°C in 5% CO_2. After washing 3 times with PBS, cells were fixed using absolute methanol for 15 minutes. The cells were washed 3 times with PBS, and stained with 5% (v/v) Giemsa stain (Life Technologies, UK) for 45 minutes at room temperature before washing 3 times by PBS. Light microscopy was used to examine the cells under oil immersion.

2.5.6.2. Invasion assay

This assay was conducted as previously described in the adhesion assay, however one step was added before staining with 5% Giemsa. After washing the cells following the infection step, infection medium containing 125 µg/ml gentamicin, which is the lethal concentration for *Cronobacter*, was added to the wells and incubated in 5% CO_2 at 37°C for 1 hour to kill the extracellular bacteria. In this assay, HMGC cell line was examined in addition to the Caco-2 and HBMEC cell lines to visualise the up-taken bacterial cells.

2.5.6.3. The effect of *C. sakazakii* infection on human cells over time

Human cells including Caco-2, HBMEC, and HMGC cell lines were grown as stated in Section 2.5.5.1, and then seeded at $4x10^4$ cell/well for 48-72 hours in 5% CO_2 at 37°C to produce confluent monolayers. *C. sakazakii* strains were prepared as explained previously in Section 2.5.5.2. The suspension was then added to the wells at $4x10^6$ cfu/well (MOI 100) for Caco-2 and HBMEC cell lines and $4x10^5$ cfu/well (MOI 10) for microglial cells. The plates then were incubated in 5% CO_2 at 37°C for 1, 3, and 5 hours. The cells were then examined using an inverted microscope.

2.6. Host response to bacterial infection

2.6.1. Human total iNOS immunoassay

This assay was performed according to manufacturer's instructions (R&D Systems Europe). HBMEC cells were grown as mentioned in Section 2.5.5.1. and then seeded into each well of a 96-well plate at a concentration of $4x10^4$ cell/well. The plate was then incubated for 48 hours at 37°C in 5% CO_2 until cells formed confluent monolayer. The following day, cells were infected with bacterial suspension for 3 hours at $4x10^6$ cfu/ml (MOI 100) prepared as described in Section 2.6.2. Then, the cells were fixed using 4% formaldehyde and incubated overnight at 2-8°C.

Afterwards, the cells were washed 3 times using the washing buffer provided by the kit manufacturer. The quenching buffer was added afterwards and the plate was incubated 20 minutes at room temperature.

After washing 3 times with the washing buffer, the blocking buffer was added and left for 1 hour at room temperature. The primary antibody mixture was added to each well after washing 3 times with the washing buffer, and incubated overnight at 2-8°C. The wells were washed and the secondary antibody was added for 2 hours at room temperature. Two substrates F1 (HRP) and F2 (AP) were added, and the plate reading was performed at two different wavelengths 450nm and 600nm, using a fluorescence plate reader (FLUOstar OPTIMA, BMG LABTECH Germany; Fig.2.2). The readings at 600_{nm} represent the total amount of iNOS, whereas the readings at 450_{nm} represent the total amount of GADPH. The two readings were used to get normalised values for the wells. Control wells with no primary antibody were included to calculate the relative fluorescence units (RFUs) by subtracting their values from sample wells to remove the fluorescence background. Normalised results were obtained by dividing the total iNOS fluorescence by the total GAPDH fluorescence in each well. The normalised duplicate readings for each sample were then averaged.
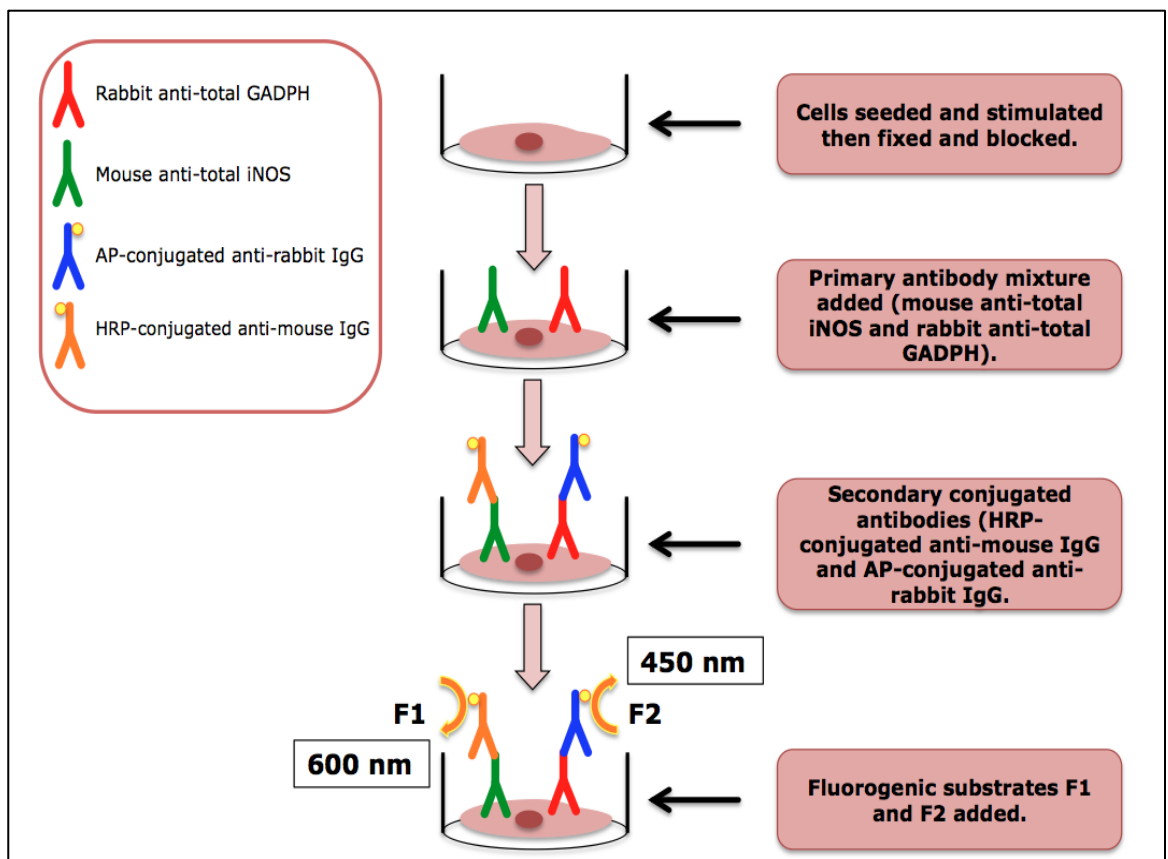
Fig.2.2 Human total iNOS immunoassay protocol using HBMEC cell line.

2.6.2. Apoptosis marker detection

This assay was conducted according to manufacturer's instructions (PromoKine, USA) with slight modifications. HBMEC and HMGC cells were seeded into 8-well BD Falcon culture slides (BD Biosciences, UK) with a concentration of $4x10^4$ cells/well. The slides were then incubated for 2 to 3 days at 37°C in 5% CO_2 to obtain confluent monolayer. After incubation, chambers were washed 3 times using PBS before inducing apoptosis by infecting cells with a bacterial suspension at $4x10^6$ cfu/ml (MOI 100) for HBMEC and $4x10^5$ cfu/ml (MOI 10) for HMGC, prepared as previously stated in Section 2.5.5.2, positive control cells were treated with 1 µM (final concentration) staurosporine (Sigma Aldrich, UK) for 3 hours to induce apoptosis. Next, the slides were incubated for 3 hours at 37°C in 5% CO_2. Afterwards, the cells were washed 3 times before staining by 1 µl DEVD-FMK conjugated to FITC for Caspase-3 activity and 5 µl of fluorescent conjugate of Annexin V-Cy3 for Annexin V activity. The cells were then washed with wash buffer provided by the kit manufacturer before fixing by 2% v/v formaldehyde and incubated overnight at 2-8°C in a dark box. Following incubation, cells were observed under a fluorescence microscope. For caspase-3 activity, FITC filter was used. Caspase-3 positive cells appear to have brighter green signals, whereas caspase-3 negative cells show much weaker signal. For annexin V activity, rhodamine filter was used. Cells that have bound annexin V showed red staining.

2.6.3. Cytokine production detection and profiling

This experiment was conducted using the Luminex® platform. The kit used in this assay was designed to measure 10 analytes simultaneously using 10-plex magnetic beads compatible with Bio-Plex® 200 platform (BIO-RAD, UK). This kit can detect 10 human cytokines including GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α.

2.6.3.1. Collecting *C. sakazakii*-infected cell lines' supernatants

HBMEC and HMGC cell lines were grown as described previously in Section 2.5.5.1. and seeded in 24-well plates at $4x10^4$ cell/well. The plates were then incubated for 48-72 hours at 37°C in 5% CO_2 to obtain confluent

monolayers. *C. sakazakii* strains' suspensions were prepared as detailed previously (refer to Section 2.5.5.2.) prior to infection. The suspension was then added to the wells at 4×10^6 cfu/well (MOI 100) for HBMEC and 4×10^5 cfu/well (MOI 10) for HMGC, and then incubated in 5% CO_2 at 37°C for 5 hours. Tissue culture medium was then collected in 1 ml eppendorfs tubes and centrifuged for 10 minutes at 13000 rpm (Mikro 200-Hettich). Next, supernatants were transferred to new 0.5 ml eppendorfs tubes and stored at -80°C until the time of the experiment.

2.6.3.2. Cytokine production

The assay was applied and the solutions were prepared according to the manufacturer's instructions (Life Technologies, UK). The plate preparation and reading were performed at Nottingham City Hospital by Dr. Ian Spendlove, Faculty of Medicine and Health Sciences, University of Nottingham. The wells of the 96-well plate were pre-wetted by adding 200 μl of working wash solution and then the liquid was removed. The 1X antibody bead solution was vortexed and sonicated and then added to each well at 25 μl/well. Next, 200 μl of working washing solution was added to each well and the beads left to soak for 30 seconds. The wells were then washed 2 times with working washing solution before adding 50 μl of incubation buffer into each well. Afterwards, 50 μl of assay diluent was pipetted into the wells followed by 50 μl of the sample. The plate was then incubated at room temperature for 2 hours with shaking on an orbital shaker at 500-600 rpm after wrapping with aluminium foil to protect the assay from light.

After incubation, the liquid was removed from wells before washing the wells 2 times with 200 μl of working washing solution. The beads were left to soak for 30 seconds. Then, 100 μl of 1X biotinylated detector antibody were added to each well and left at room temperature for one hour with shaking at 500-600 rpm. The liquid was then removed from wells before washing the wells 2 times by 200 μl of working washing solution, and the beads were left to soak for 30 seconds. Next, 100 μl of 1X streptavidin-labeled with R-Phycoerythrin (RPE) were pipetted into each well and the plate was incubated for 30 minutes at room temperature with shaking at

500-600 rpm. Afterwards, the liquid was removed from wells and the wells were washed 2 times with 200 µl of working washing solution. The beads were allowed to soak for 30 seconds. The liquid was then replaced by 125 µl of working washing solution in each well before shaking the plate on an orbital shaker for 2-3 minutes at 500-600 rpm to re-suspend the beads. Next, the plate was uncovered and inserted into Bio-Plex® 200 to analyse the samples. The results are presented as pg/ml.

2.7. Statistical analysis

Data were assessed for normality using Kolmogorov-Smirnov test and normality histograms. The normally distributed data were analysed using the parametric One-way Analysis of Variance test (ANOVA) with Tukey's post-hoc test, and were expressed as mean values and the standard error of mean (Mean±SEM). Data that were not normally distributed were subjected to Kruskal-Wallis test, the non-parametric equivalent of the parametric ANOVA, and were expressed as mean values and the standard deviation (Mean±SD). Tukey's post-hoc analysis was performed as a single step multi-comparison test to compare the significance of the means of every *C. sakazakii* strain in relation to other strains as pairwise comparisons. A *P*-value of <0.05 was considered statistically significant.

For the motility, serum resistance, attachment, invasion, and translocation assays, data analysis was performed using Kruskal-Wallis test, as their data were not normally distributed and were difficult to transform. On the other hand, MTT, persistence in macrophages, persistence in microglia, and cytokine production assays were found to be normally distributed and therefore were analysed using ANOVA. Computer statistical analysis software was used to perform the analysis (IBM SPSS version 22.0, Chicago, IL, USA).

Chapter 3: *Cronobacter sakazakii* virulence

3.1. Introduction

The colonisation of the mucosa by Gram-negative bacteria begins immediately after birth. 52-83% of neonates in neonatal intensive care units (NICUs) become colonised by these organisms 1-2 week post delivery. It was reported that most infants in NICUs were found to be colonised with Gram-negative rods including *E. coli*, *Klebsiella*, *Enterobacter*, and *Citrobacter* (Almuneef *et al.* 2001, Parm *et al.* 2011). *C. sakazakii* is an enteric Gram-negative pathogen that can be found in PIF and associated with NICU infections. It can cause NEC, bacteraemia, and meningitis resulting in a 40-80% mortality rate among infected infants and 20% of the survivors also develop serious neurological disorders (Bowen and Braden 2006, Mange *et al.* 2006, Caubilla-Barron *et al.* 2007, Giri *et al.* 2011). In 1994, 3 infants died in a NICU in France as a result of *C. sakazakii* infections. They suffered from different forms of infection including NEC (i.e. strain 695) and meningitis (i.e. strain 767; Caubilla-Barron *et al.* 2007).

For some organisms to establish an infection they must adhere to the host cell, translocate to the underlying tissues, and then disseminate throughout the body. The epithelium has an important role in protecting the body against bacterial invasion. Once this layer loses its integrity the invading organism will find its way to infect the tissue beneath (Wilson *et al.* 2002). The ability of *C. sakazakii* to invade the intestinal epithelium and brain endothelium is considered as a crucial step for pathogenesis. It was shown previously that the organism has the ability to produce an enterotoxin and to adhere to epithelial and endothelial cells *in vitro* such as Caco-2 and HBMEC cell lines (Pagotto *et al.* 2003, Mange *et al.* 2006). A study by Townsend *et al.* (2008) used a group of isolates from the French outbreak in 1994, and showed that *C. sakazakii* strains are able to adhere and invade Caco-2 and rBCEC4 cell lines. Moreover, the organism was able to persist and multiply within the human macrophage U937 cell line (Townsend *et al.* 2007b). Furthermore, another study by Giri *et al.*

(2011) showed that some *C. sakazakii* strains have the ability to invade INT407, Caco-2, and HBMEC cell lines.

Translocation of the organism follows the attachment and invasion. It is the step that initiates pathogenesis in the next levels of tissues after passing through the epithelium layer. It was reported by Townsend *et al.* (2007a) that the presence of LPS in PIF increases the permeability of tissue barriers leading to the translocation of intestinal bacteria including *C. sakazakii*. Giri *et al.* (2011) showed that the invasive *C. sakazakii* strains were able to translocate through the intact monolayers of Caco-2 and HBMEC cell lines. This suggests that the bacterium is able to overcome the physical host barriers in the intestines and CNS.

Attachment, invasion, and translocation need bacterial virulence traits such as motility, iron acquisition, serum resistance, and cytotoxicity that could assist the organism to overcome host barriers. The cytotoxicity of the bacterium might collaborate in the translocation through the intact monolayers of the cell lines by inducing cell death, which leads to cell line permeability. Cytotoxicity assays are commonly used for *in vitro* assessment of mammalian cell viability following exposure to a toxic substance, and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) transformation is one of the assays that is used widely to assess cell viability. MTT is a water-soluble tetrazolium salt that can be transformed to its insoluble purple formazan form. This is as a result of the reduction of the tetrazolium ring by succinate dehydrogenase inside mitochondria. Formazan is impermeable and therefore can not cross the cell membrane, and this leads to the accumulation of formazan crystals in healthy cells. On the other hand, dead cells that have lost their membrane integrity lose formazan and display a less intense purple colour as a result of low MTT reduction (Fotakis and Timbrell 2006, Sharma *et al.* 2009, Travan *et al.* 2009).

Several genes have been linked to the virulence of *Cronobacter* species. However, the severity of virulence among strains is varied. These genes encode many virulence factors including those involved in adhesion and invasion of epithelial cells, persistence in macrophages, motility, iron

acquisition, and serum resistance (Bowen and Braden 2006, Kucerova *et al.* 2010, Franco *et al.* 2011a, Cruz-Córdova *et al.* 2012). A recent study based on whole genome sequencing revealed that *C. sakazakii* BAA-894 carries two plasmids; pESA2 and pESA3 (Kucerova *et al.* 2010, Joseph *et al.* 2012b). Many virulence gene clusters are encoded on the large plasmid pESA3 including two iron acquisition loci; *eit*CBAD and *iuc*ABCD/*iut*A. Moreover, pESA3 encodes the *viuB* gene that that has a putative role in the aerobactin siderophore system and might have a role in serum resistance in addition to the product of *cpa* gene (Bogard and Oliver 2007, Grim et al. 2012). Iron has a vital role in bacterial pathogenesis. At the time of infection, the innate immune system in the human body reduces the free iron levels, which leads to the inhibition of bacterial growth. This drives the bacteria to produce iron siderophores, which in turn scavenge iron from iron-limited environments (Franco *et al.* 2011a, Grim *et al.* 2012).

It was also reported that pESA3 encodes for the outer membrane protease Cpa, which is responsible for serum resistance. This protease of *Cronobacter* provides resistance against complement-dependent killing of serum by cleaving complement components C3 and C4b. Moreover, it has a major role in converting plasminogen to plasmin, which leads to the activation of other proteolytic enzymes, including matrix metalloproteinases, resulting in degradation of the tight junctions of microvascular endothelial cells. This will allow the bacteria to migrate to peripheral tissue and invade the CNS (Lähteenmäki *et al.* 2005, Franco *et al.* 2011b). Avoiding serum-mediated killing together with the persistence within macrophages and other phagocytic cells gives the organism an advantage so it can survive in the blood stream, multiply, cause bacteraemia, and potentially reach vital organs such as the brain and the meninges. Furthermore, degrading the components of the tight junctions will result in the migration of the bacterial cells leading to more damage to the infected organ or tissue.

With regard to motility, the flagellum is the primary bacterial organelle that is responsible for motility and chemotaxis. Moreover, it can play

several roles other than motility including aiding adhesion, and biofilm formation (Hartmann *et al.* 2010, Amalaradjou and Venkitanarayanan 2011). *C. sakazakii* is a motile bacterium that uses peritrichous flagella for motility. It was shown recently that the flagella of this organism contribute in the attachment to Caco-2 cells and collaborate in biofilm formation (Hartmann *et al.* 2010). Moreover, *C. sakazakii* mutants that lacked flagella or had shorter flagella showed reduced biofilm formation and decreased adhesion to Caco-2 cells (Hartmann *et al.* 2010, Haiko and Westerlund-Wikström 2013). Furthermore, flagella are able to trigger the production of the pro-inflammatory cytokines such as IL-8 and TNF-α in addition to the anti-inflammatory cytokine IL-10 in monocytes, polymorphonuclear, dendritic, and epithelial cells (Honko and Mizel 2005, Cruz-Córdova *et al.* 2012). Hence, flagella expression is a very important virulence factor, which aids the adhesion to surfaces and mammalian cells in addition to their role in triggering the host immune responses. Moreover, the genome analysis of *C. sakazakii* revealed that the organism encodes *ompA* and *ompX* genes that were found to promote invasion of Caco-2 and HBMEC cell lines by *Cronobacter* spp. (Mohan Nair *et al.* 2009, Kim *et al.* 2010, Joseph *et al.* 2012b).

A multilocus sequence typing scheme (MLST) of *Cronobacter* spp. has been constructed, initially for *C. sakazakii* and *C. malonaticus*, by Baldwin *et al.* (2009) which now covers all seven *Cronobacter* species and is available online at http://www.pubMLST.org/cronobacter. Joseph and Forsythe (2011) investigated the association between the severity of the infection and the sequence type (ST) profile. That study found that ST4 contains the most virulent strains that caused fatal neonatal NEC and meningitis infections including the French outbreak strains 695 and 767. A follow-up study by Joseph and Forsythe (2012c) using eBURST analysis showed that *Cronobacter* genus exhibited 13 single locus variant clonal complexes among the 115 identified STs. The clonal complex where strains are identical in 3 or more loci, and *C. sakazakii* is represented in nine of these clades (Forsythe *et al.* 2014). Clonal complex 4 (CC4) contains *C. sakazakii* STs 4, 15, 97, 107 108, and 109, and as mentioned previously ST4 is the most frequent clinical ST (Forsythe *et al.* 2014).

Another study by Hariri *et al.* (2013) examined 15 *Cronobacter* isolates that had been received from CDC. These strains were isolated in 2011 in the United States. The study found that 5 CSF isolates were either belonging to ST4 or CC4. This supports the previous conclusion (Joseph and Forsythe 2011, Joseph and Forsythe 2012c) that *C. sakazakii* CC4 is the predominant clonal complex in CSF isolates among *Cronobacter* species. Therefore, it is important to investigate the virulence potential of this clonal complex and to compare it with other sequence types of *C. sakazakii*.

Kim (2003) reported the possible pathogenic pathway of bacterial-induced meningitis for several organisms including *E. coli* K1. Based on this review, the hypothesis of this project with regard to the pathogenic pathway is that *C. sakazakii* adheres, invades and then translocates through the intestinal epithelium to the underlying tissue and blood stream. Then, it persists, multiplies, and disseminates throughout the body causing bacteraemia that could lead to septicaemia. Subsequently, it can attach, invade, and then translocate through the brain endothelium and cross the blood brain barrier (BBB) to cause meningitis.

C. sakazakii has many potential virulence factors that allow the bacterium to grow, survive, cause infections, and support pathogenesis. The presence of these factors has major implications on the host that might lead to severe outcomes. The aim of this chapter is to examine the virulence factors of interest that help the organism to establish a successful infection and avoid host protective responses. Moreover, it aims to investigate the ability of *C. sakazakii* strains, especially clinical strains, from different sequence types including CC4 to adhere, invade and translocate through a group of cell lines including Caco-2, HBMEC, rBCEC4 cell lines. Moreover, it will discuss their potential to persist and replicate within phagocytic cells using U937 and HMGC cell lines in addition to their cytotoxicity. The experiments in this chapter well help to understand the pathogenic pathway of *C. sakazakii*-induced meningitis by using clinical strains that were isolated from meningitis cases and CSF isolates. These strains are expected to be invasive and able to translocate

through different cell lines in addition to their ability to avoid phagocytosis and serum-mediated killing by the host.

3.2. Materials and Methods overview

A group of *C. sakazakii* strains were tested for their virulence potential and their ability to overcome host barriers. The motility test was applied to investigate the ability of *C. sakazakii* clinical strains to move, which might prove the presence of flagella. The plasmid of the test strains was extracted and examined for the presence of the iron acquisition and serum resistance genes. Moreover, genome comparisons using Artemis, WebACT, and BLAST search were used to confirm the presence and the absence of virulence genes of interest in the sequenced strains. Iron siderophore production using CASAD and the sensitivity to human serum assays were conducted to confirm the ability of the bacterium to produce iron siderophores and to survive in human confirming these activities in the presence of the genes that might be responsible for these phenotypes.

Different tissue culture techniques were used in this chapter, and the aim was to examine the ability of the organism to overcome the host barrier at different levels starting from the gut epithelium layer, disseminating to the blood stream, and reaching the CNS. The MTT test was applied to clarify the cytotoxic effect of *C. sakazakii* strains on host cells *in vitro*. Moreover, *C. sakazakii* clinical strains including CC4 meningitis and CSF isolates were tested for their ability to attach and invade Caco-2, HBMEC, and rBCEC4 cell lines. In addition, they were examined for their translocation potency through the intact monolayers of Caco-2 and HBMEC cell lines. Furthermore, their ability to survive and multiply within phagocytic cells was examined. Additionally, the integrity of the monolayer cell lines was tested using the TEER measurement. The methods, media, chemical reagents, cell line maintenance, and culture preparation were described previously in Chapter 2 (Materials and methods).

For the statistical analysis, data were assessed for normality using Kolmogorov-Smirnov test and normality histograms. The normally

distributed data were analysed using the parametric One-way Analysis of Variance test (ANOVA) with Tukey's post-hoc test, and were expressed as mean values and the standard error of mean (Mean±SEM). Data that were not normally distributed were subjected to Kruskal-Wallis test, the non-parametric equivalent of the parametric ANOVA, and were expressed as mean values and the standard deviation (Mean±SD). Tukey's post-hoc analysis was performed to compare the significance of the means of every *C. sakazakii* strain in relation to other strains as pairwise comparisons. A *P*-value of <0.05 was considered statistically significant. For the motility, serum resistance, attachment, invasion, and translocation assays, data analysis was performed using Kruskal-Wallis test, as their data were not normally distributed and were difficult to transform. On the other hand, MTT, persistence in macrophages, and persistence in microglia assays were found to be normally distributed and therefore were analysed using ANOVA.

3.3. Results

3.3.1. *C. sakazakii* motility

3.3.1.1. Motility test

The experiment was repeated two times in triplicate. The motility of *C. sakazakii* strains was measured by the diameter of growth around the inoculation area in millimetres (mm) in the motility medium as described in Section 2.5.1. Varying motility zones were recorded for the organism (see Fig.3.1). All *C. sakazakii* strains were motile when inoculated in soft agar except for strains 1223 (ST4), 1224 (ST4), and 680 (ST8), which showed significantly smaller migration zones (<5 mm; *P*<0.001), and were considered as non-motile. CC4 Strains 695, 721, 767, and 1587 in addition to strains 1249 (ST31) 696 (ST12) showed highest motility (zone diameter >15 mm). The other strains demonstrated a lower motility ability with a zone diameter <15 mm. Refer to Table.3.3 for summary of results.
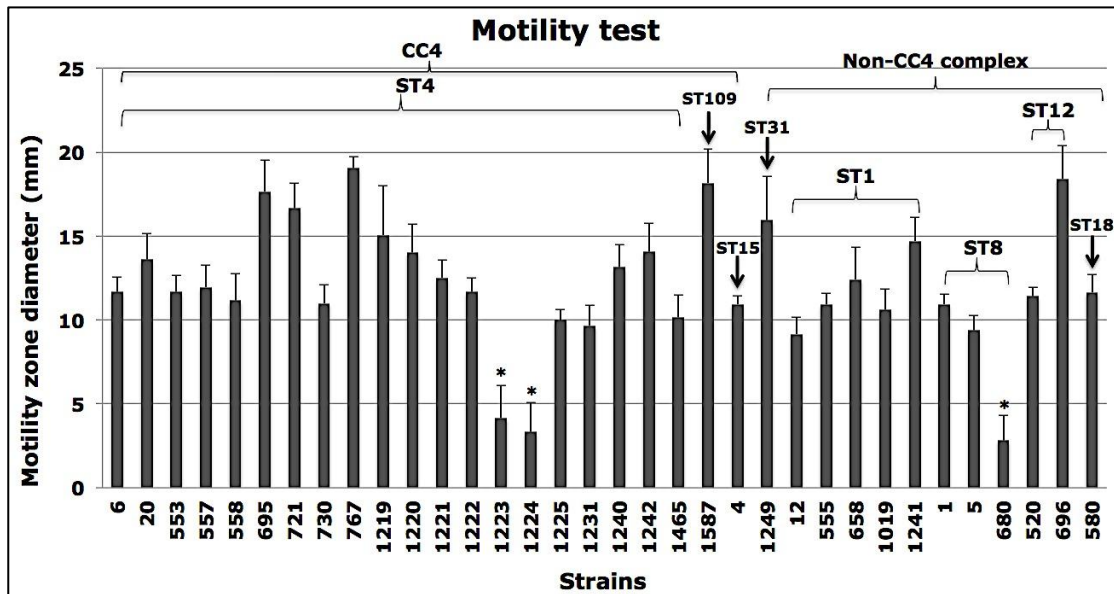
Fig.3.1 *C. sakazakii* motility test after overnight incubation at 37°C in soft agar. The diameters of the motility zones were measured in mm, and the data are presented as the mean±standard deviation of the replicates of two independent experiments. The asterisks above the bars indicate statistically significant differences between *C. sakazakii* strains in this experiment (*$P<0.001$; Kruskal-Wallis).

ST: Sequence type.
CC4: clonal complex 4.

3.3.1.2. Flagellar genes

A BLAST genome search was applied to test the presence of *fli* genes that are linked to flagella expression and movement in *C. sakazakii* (Kucerova *et al.* 2010, Joseph *et al.* 2012b). All strains in this project (*n*=24) that have their genomes sequenced showed the presence of *fliA*, *fliC*, *fliD*, *fliS*, *fliT*, *fliY*, and *fliZ* genes (Table.3.1). Ninety two percent of the strains shared the presence of *fliN*, *fliO*, *fliP*, and *fliQ* whereas strains 680 (ST8) and 520 (ST12) were negative for these genes. All strains were positive for *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliK*, *fliL*, and *fliM* except for the non-motile strains 680 (ST8) that lacked these genes. This suggests that the annotated flagellar genes that are absent in the genome of strain 680 and present in the genomes of the motile strains are essential for flagella expression and movement.

Table.3.1: Presence/absence of flagellar genes in sequenced genomes of *C. sakazakii* strains.

Strains	ST	Motility	fliA	fliC	fliD	fliE	fliF	fliG	fliH	fliI	fliJ	fliK	fliL	fliM	fliN	fliO	fliP	fliQ	fliR	fliS	fliT	fliY	fliZ	Genome sequenced
6	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
20	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
553	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
557	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
558	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
695	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
721	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
730	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
767	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1219	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1220	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1221	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1225	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1231	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1240	4	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1587	109	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
4*	15	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
1249	31	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
658	1	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	2
1	8	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
5	8	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1
680	8	No	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	3
520	12	Yes	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	1
696	12	Yes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3

Flagellar gene loci; **fliA:** ESA_01300. **fliC:** 01288. **fliD:** ESA_01287. **fliE:** 01261. **fliF:** ESA_01260. **fliG:** ESA_01259. **fliH:** ESA_01258. **fliI:** ESA_01257. **fliJ:** ESA_01256. **fliK:** ESA_01255. **fliL:** ESA_01254. **fliM:** ESA_01253. **fliN:** ESA_01252. **fliO:** ESA_01251. **fliP:** ESA_01250. **fliQ:** ESA_01249. **fliR:** ESA_01248. **fliS:** ESA_01286. **fliT:** ESA_01285. **fliY:** ESA_01302. **fliZ:** ESA_01301.
ST: Sequence type. **Yes:** Motile. **No:** Non-motile. **+:** Present. **-:** Absent.
*: Part of clonal complex 4, which contains STs 4, 15, 97, 107, and 108 (Joseph *et al.* 2012d).
1: Exeter. **2:** Washington. **3:** Life Technologies.

3.3.2. Iron acquisition

3.3.2.1. Iron siderophore detection using CASAD

Almost all *C. sakazakii* strains were able to produce iron siderophores with CAS agar showing orange halo around the wells, however strains 6 (ST4) and 520 (ST12), the plasmid-less strains, were negative (*P*<0.001). Strains 1223 (ST4) and 1 (ST8) produced the highest siderophore levels based on the orange halo diameter measurements of 21 and 21.5 mm respectively (Fig.3.2). The rest of the strains produced halo diameters between 9 and 18.75 mm (Fig.3.2). The results are summarised in Table.3.3.
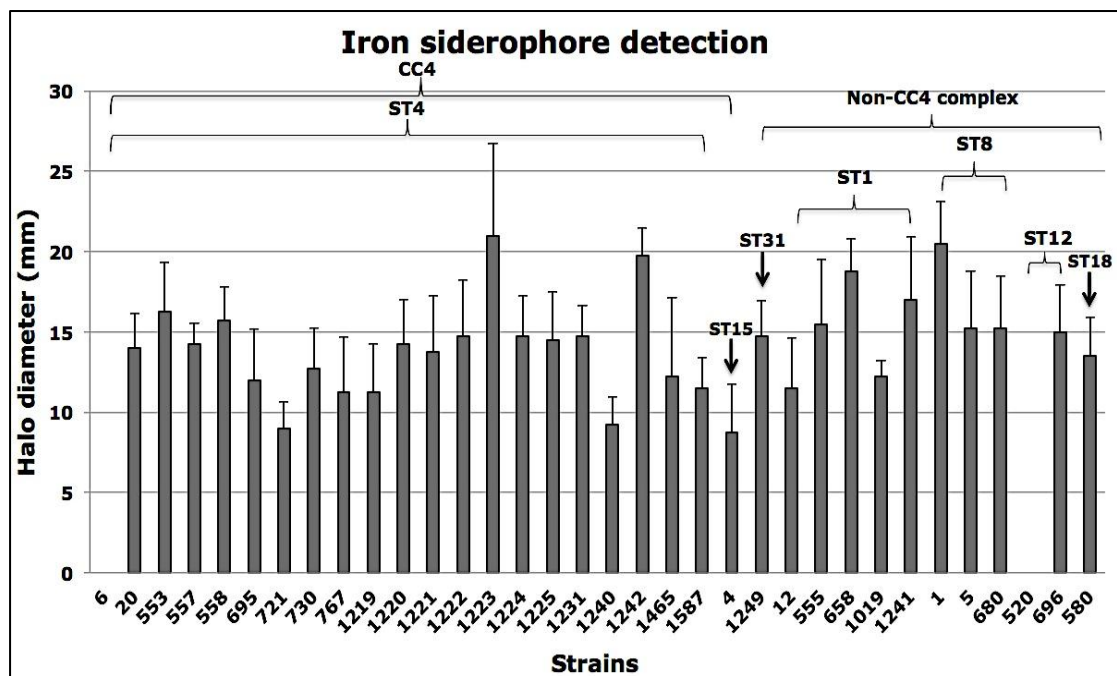
Fig.3.2 Iron siderophore production by *C. sakazakii* strains using the CASAD assay. Results presented as mean±standard deviation of two independent experiments.

ST: Sequence type.
CC4: clonal complex 4.

3.3.2.2. Iron acquisition genes

The two iron acquisition loci *eit*CBAD and *iuc*ABCD/*iut*A are encoded on the pESA3 plasmid of *C. sakazakii* strains. These were investigated according to Franco *et al.* (2011a) using PCR primers for *eitA* and *iucC* genes. The results are displayed in Table.3.2. The gene *eitA* is a

component of a transporter whose homologues are involved in transport and promote the translocation of ferric iron, siderophores, and haem, while *iucC* is a part of *iucABCD* iron uptake system, which is responsible for cronobactin biosynthesis and transport, that mediates *Cronobacter* spp. growth under iron limiting conditions (Franco *et al.* 2011a, Grim *et al.* 2012). Ninety one percent of *C. sakazakii* strains (*n*=34) were positive for *eitA* and *iucC*. The presence of *eitA* and *iucC* genes was confirmed in 91% and 95% respectively of CC4 strains (*n*=22). With regard to ST1, all strains were *eitA* positive while 80% were positive for the *iucC* gene. All ST8 strains in addition to strains 1249 (ST31), 696 (ST12) and 580 (ST18) were positive for both genes. On the other hand, the plasmid-less strains 6 (ST4) and 520 (ST12) lack both of the iron acquisition regions. All strains that were positive for iron acquisition genes showed positive results for iron siderophore production (Fig.3.2). Strain 557 (ST4) was negative for *eitA* and positive for *iucC*, and strain 12 (ST1) was positive for *eitA* and negative for *iucC*. This, however, did not affect their ability to produce iron siderophores, and this might be attributed to the presence other siderophore-related genes that were not examined in this project (Table3.2 and Fig.3.2).

In addition to PCR, the genomes of the sequenced strains were explored for the presence of iron acquisition genes (Table.3.2). Eighty eight percent of the sequenced strains in this project (*n*=24) encoded the *eitA* gene, while 92% encoded *iucC*. Strain 557 (ST4) lacked the presence of *eitA* gene. The plasmid-less strains 6 (ST4) and 520 (ST12) lacked *eitA* and *iucC* genes. Moreover, 88% of these strains were positive for *viuB* gene that has a putative role in the aerobactin siderophore system according to Grim *et al.* (2012). However strains 6 (ST4), 520 (ST12), and 696 (ST12) were negative. Strains 6 (ST4), 520 (ST12) lacked the presence of the plasmid and did not show the presence of the iron acquisition genes in their genomes. This is in agreement with the results that were obtained previously in the iron siderophore detection assay using CASAD, as the strains were not able to produce iron siderophores (Fig.3.2).

3.3.3. Serum resistance

3.3.3.1. *C. sakazakii* sensitivity to human serum

The majority of *C. sakazakii* strains in this experiment (n=13) showed up to 60% decrease in their viable count after the first hour of incubation with human serum except for strain 558 (ST4) that demonstrated a slight increase. After the second hour of incubation, it was noted that 85% of the strains showed an increase in their numbers, however strains 6 (ST4) and 680 (ST8) displayed a decrease in their numbers showing 20-80% reduction in their viability. The vast majority of the strains from different sequence types were able to survive in human serum and showed significant increases of upto 400% in their initial numbers after the third hour of incubation. Strains 20 (ST4), 1242 (ST4), 1249 (ST31), and 696 (ST12) showed highest serum tolerance. In contrast, strains 6 (ST4) and 680 (ST8) were serum sensitive and their viability in serum declined over the assay period (P<0.05).

S. Enteritidis and *Cit. koseri*, which were used as the positive control strains for tissue culture experiments, showed increased growth yields indicating their tolerance to human serum. On the other hand, *E. coli* K12, which was the negative control strain, exhibited reduced growth levels, which is a sign of serum sensitivity (P<0.05; Fig.3.3). A summary of the previous results is provided in Table.3.3.
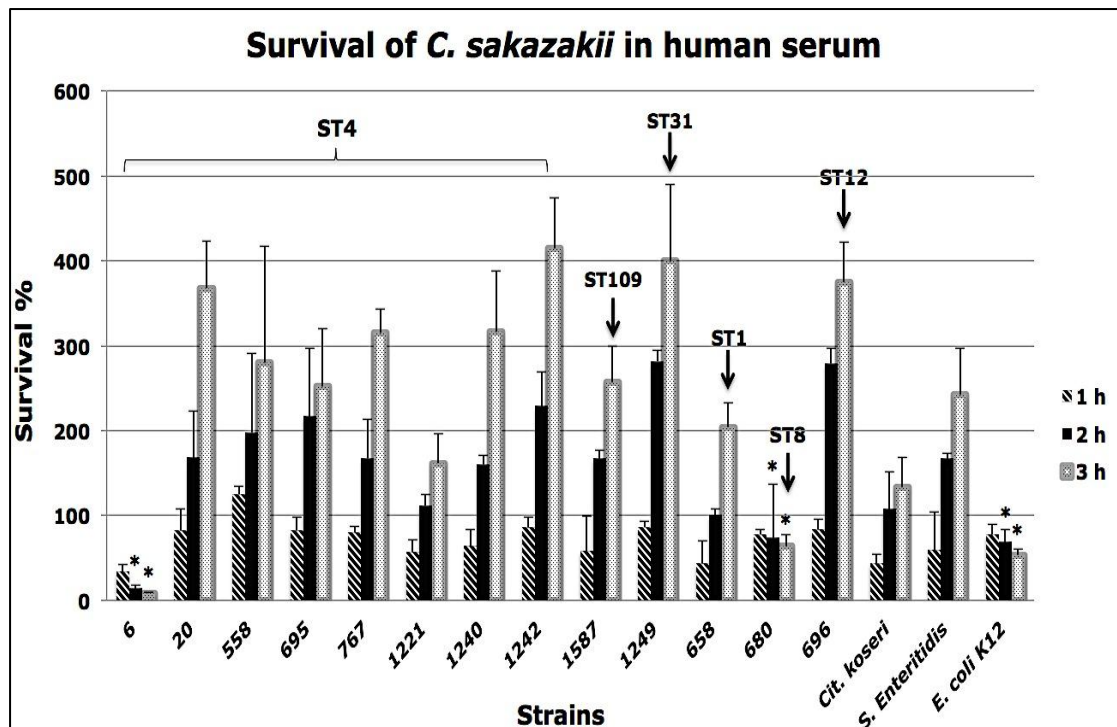
Fig.3.3 Sensitivity of *C. sakazakii* to human serum over 3 hours of incubation showing the difference in growth among strains overtime. Most of the strains showed increases in their viable counts including *Cit. koseri* and *S*. Enteritidis, and strains 6 (ST4), 680 (ST4), and *E. coli* K12 showed significantly reduced viability. The displayed data are the mean±standard deviation of survival % (10^6 cfu/ml initial inoculum) compared to time 0 of two independent experiments. The asterisks above the bars indicate statistically significant differences between *C. sakazakii* strains in this experiment (*$P<0.05$; Kruskal-Wallis).

ST: Sequence type.

3.3.3.2. Genes responsible for serum resistance

Based on the research of Franco *et al.* (2011b), the outer membrane protease Cpa of *Cronobacter* is responsible for serum resistance. All *C. sakazakii* strains were tested for the presence of the *cpa* gene. By PCR 85% of the test strains (*n*=34) were confirmed positive for this gene. The vast majority of CC4 strains (86%; 19 out of 22 strains) harboured the *cpa* gene. All ST1 strains (*n*=5) encoded the gene in addition to strains 1249 (ST31), 696 (ST12) and 580 (ST18). Regarding ST8 (*n*=3), 2 strains were positive for the *cpa* gene and strain 680 was negative. Moreover, Strains 6 (ST4) and 520 (ST12) were negative for this gene as they lack the pESA3 plasmid (Table.3.2). With regard to the genomes of the sequenced strains, 88% of the strains (*n*=24) were *cpa* positive, and strains 6 (ST4), 680 (ST8), and 520 (ST12) were negative (Table.3.2).

The regulator genes of capsule synthesis encoded in the locus *rcs* were reported to control exopolysccharide colanic acid production. These genes including *rcsA*, which was considered as a positive regulator of colanic acid biosynthesis, have been identified previously in *E. coli* K12 (Gottesman *et al.* 1985, Allen *et al.* 1987). It was reported that the mucoid appearance of *E. coli* K12 at 37°C was attributed to the presence of the *rcsA* gene product (McCallum and Whitfield 1991). It has been shown recently that colanic acid provides protection against the bactericidal effect of human serum in *E. coli* (Li *et al.* 2005, Miajlovic *et al.* 2014). Moreover, colanic acid can be used by serum-resistant *E. coli* strains as a shield while repairing the damaged to the cell wall caused by bactericidal serum factors (Miajlovic and Smith 2014). It was found that about 92% of *C. sakazakii* sequenced strains (*n*=24) that were used in this research have *rcsA* gene in their genomes (Table.3.2). Fifty four percent of these strains (*n*=13) were tested for serum resistance and 84% (*n*=11) of them were resistant to human serum killing (Fig.3.3). Strain 6 (ST4) that was positive for *rcsA* and negative for *cpa* showed serum sensitivity. In addition, strain 680 (ST8) that lacked both *cpa* and *rcsA* genes was serum sensitive. These results suggest that *cpa* has an important role in serum resistance and its absence could affect the ability of the bacterium to survive human serum-mediated killing.

Table.3.2: Presence/absence of virulence genes on the pESA3 plasmid and the sequenced genomes of *C. sakazakii* strains.

Strains	ST	PCR probes			Genomic search																		Genome sequenced
		eitA	*iucC*	*cpa*	*eitA*	*iucC*	*viuB*	*cpa*	*rcsA*	*apaH*	*ompA*	*ompX*	*ygdP*	*ppk1*	*phoP*	*phoQ*	*pmrA*	*pmrB*	*pmrE*	*mgtB*	*gsrA*	*sodA*	
553	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
695	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
730	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
767	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
557	4	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
721	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1222	4	+	+	+																			
1223	4	+	+	-																			
1224	4	+	+	-																			
1242	4	+	+	+																			
1465	4	+	+	+																			
4**	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (2)
12	1	+	-	+																			
555	1	+	+	+																			
1019	1	+	+	+																			
1241	1	+	+	+																			
1	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (1)
680	8	+	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (3)
696	12	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Y (3)
580	18	+	+	+																			
6*	4	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	Y (1)
520*	12	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	Y (1)

ST: Sequence type. **+:** Present. **-:** Absent. **N/S:** Not sequenced. ***:** Plasmid-less strains. **Y:** Yes. **(1):** Exeter. **(2):** Washington. **(3):** Life Technologies.
**: Part of clonal complex 4, which contains STs 4, 15, 97, 107, 108, and 109 (Forsythe *et al.* 2014).
Genes' loci, **eitA:** ESA_pESA3p05518. **iucC:** ESA_pESA3p05549. **viuB:** ESA_pESA3p05545. **cpa:** ESA_pESA3p05434. **rcsA:** ES15_1478. **apaH:** ECs0054. **ompA:** ESA_02391. **ompX:** ESA_02526. **ygdP:** APECO1_3675. **ppk1:** APECO1_4068. **phoP:** STM1231. **phoQ:** STM1230. **pmrA:** ESA_03574. **pmrB:** ESA_03575. **pmrE:** ESA_01534. **mgtB:** STM3763. **gsrA:** YPO3382. **sodA:** ESA_03843.

3.3.5. Tissue culture cell-based virulence studies

3.3.5.1. *C. sakazakii* cytotoxic activity to human cell lines (MTT)

Cytotoxicity is crucial for bacteria to induce cell death in eukaryotic cells. By causing cell death, bacteria can disrupt the tight junctions of the polarised cell lines leading to their translocation to the underlying tissues. . *C. sakazakii* strains were tested for their ability to induce cytotoxicity for Caco-2, HBMEC, and HMGC cell lines using the MTT test (Fig.3.4), where the viability of eukaryotic cells was indicated by absorbance OD_{600}. In this experiment an additional washing step with PBS was added before adding the MTT to minimise the number of extracellular bacterial cells in the wells to avoid false positive results.
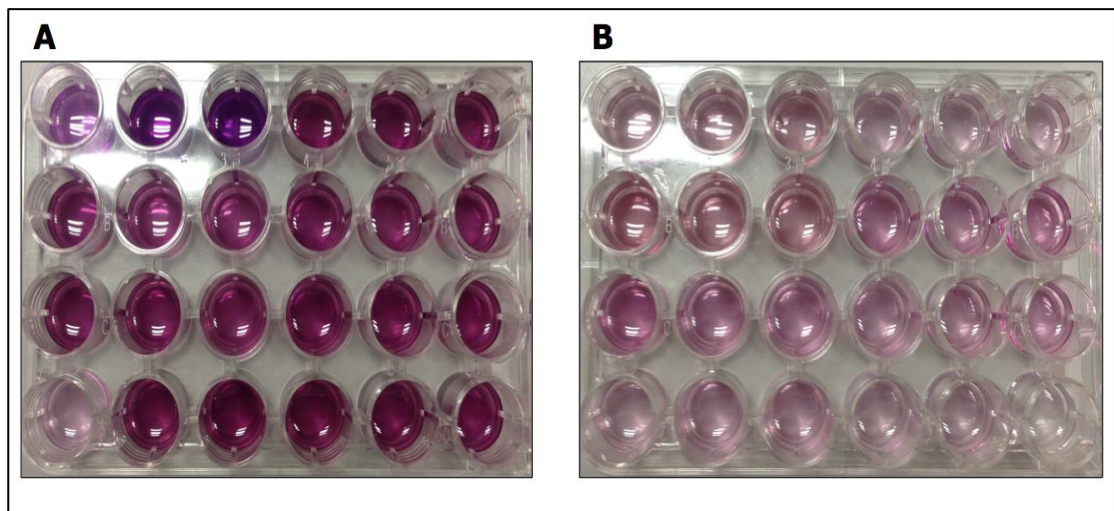
Fig.3.4 Cytotoxicity assay using MTT method. The example pictures show the effect of *C. sakazakii* strains on the Caco-2 cell line over time. **(A)** The patterns of MTT reduction 1 hour post infection showing accumulated purple colour in the vast majority of wells indicating that the cells are viable. **(B)** After 3 hours of infection, MTT reduction levels declined as shown by a pale purple colour indicating increasing cell death as a result of the infection.

For the Caco-2 cell line, most of the strains showed MTT reduction 1 hour after infection. However strains 6 (ST4), 558 (ST4), 680 (ST8), and *E. coli* K12 (the negative control for tissue culture experiments) were low in cytotoxicity (showed high levels of MTT reduction) as low as the assay's negative control (uninfected cells). Strains 20 (CC4), 695 (CC4), 1587 (CC4), and 658 (ST1) demonstrated significantly higher cytotoxicity levels (lower MTT reduction levels) more than other tested strains ($P<0.05$).

Compared to 1-hour infection period, after 3 hours of infection some changes in cytotoxicity were noticed. Strains 20 (CC4), 1242 (CC4), 1587 (CC4), 658 (ST1), and *S.* Enteritidis (tissue culture positive control) caused significantly lower MTT conversion (*P*<0.05) indicating their high cytotoxic ability (Fig.3.5). Although most CC4 strains showed high cytotoxicity, strains 6 and 558 were less cytotoxic suggesting that they have low virulence potential that might affect their ability of translocating through mammalian cell lines. The cytotoxic activity is crucial in the translocation process through this cell line, which will be discussed later in this chapter, and it appears to be due to the attachment and invasion ability of the organism.

Fig.3.5 Cytotoxicity of *C. sakazakii* strains on Caco-2 up to 3 hours of incubation. MTT reduction was used to measure the cytotoxicity levels of *C. sakazakii* strains where only the viable Caco-2 cells are able to reduce MTT to its insoluble purple form formazan, the higher absorbance (OD$_{600}$) the higher in MTT reduction (low toxicity) and vice versa. The negative control used was uninfected cells treated using the same protocol with no bacteria added. The data presented in mean±standard error of mean of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*P*<0.05; ANOVA).
ST: Sequence type.

With regard to the HBMEC cell line, it showed resistance to the cytotoxicity of *C. sakazakii* in addition to that of *S*. Enteritidis, *Cit. koseri*,

and *E. coli* K12 over 1 hour of infection. The absorbance in most wells remained high or demonstrated a slight decrease when compared with the assay's negative control. However, strain 1249 (ST31) and *Cit. koseri* produced the highest cytotoxicity values. On the other hand, a prolonged infection period for 3 hours led to an increase in cytotoxicity with 69% of strains. CC4 strains 20, 695, 767, 1221, 1240, and 1587 in addition to 658 (ST1), 696 (ST12), and *Cit. koseri* showed significantly higher cytotoxicity ($P<0.05$), while strains 6 (CC4), 558 (CC4), 1242 (CC4), 680 (ST8), and *E. coli* K12 caused the lowest cytotoxicity (Fig.3.6). The ability of this cell line to be more resistant to the cytotoxicity of *C. sakazakii* for a prolonged incubation period might play an essential role in translocation resistance by maintaining the integrity of the cell monolayer. This will be discussed later in this chapter.

Fig.3.6 Cytotoxicity of *C. sakazakii* strains on HBMEC up to 3 hours of incubation. MTT reduction was used to measure the cytotoxicity levels of *C. sakazakii* strains where only the viable HBMEC cells are able to reduce MTT to its insoluble purple form formazan, the higher absorbance (OD_{600}) the higher in MTT reduction (low toxicity) and vice versa. The negative control used was uninfected cells treated using the same protocol with no bacteria added. The data presented in mean±standard error of mean of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$; ANOVA).
ST: Sequence type.

For the HMGC cell line, 1 hour after infection there was no significant increase compared to the assay's negative control in the cytotoxicity for the strains 6 (CC4), 558 (CC4), 1242 (CC4), 658 (ST1), 680 (ST8), and *E. coli* K12, the negative control for tissue culture experiments, showing high absorbance levels with values nearly similar to the assay's negative control indicating that the human cells were viable and able to reduce MTT. Nonetheless, CC4 strains 20, 695, 767, 1221, 1240, 1587 in addition to strains 1249 (ST31), 696 (ST12), and *Cit. koseri* (the positive control for tissue culture experiments) showed a significant effect on HMGC cells showing the highest cytotoxicity among strains ($P<0.01$). On the other hand, when the infection period was extended to 3 hours, increased cytotoxicity levels were recorded indicating an increase in HMGC cell death. CC4 strains 20, 695, 767, 1221, 1240, and 1587 in addition to *Cit. koseri* exhibited the highest cytotoxicity activity ($P<0.001$). Strains 6 (CC4), 558 (CC4), 680 (ST8), and *E. coli* K12 maintained a low level of cytotoxicity, while strains 1242 (CC4), and 658 (ST1) were moderately cytotoxic (Fig.3.7). CC4 strains 6 and 558 were the lowest in cytotoxicity among all strains from the same sequence type. Cytotoxicity for this kind of cell might help the organism to avoid phagocytosis and induce cell death, which leads to its survival. The previous results are summarised in Table.3.4 below.
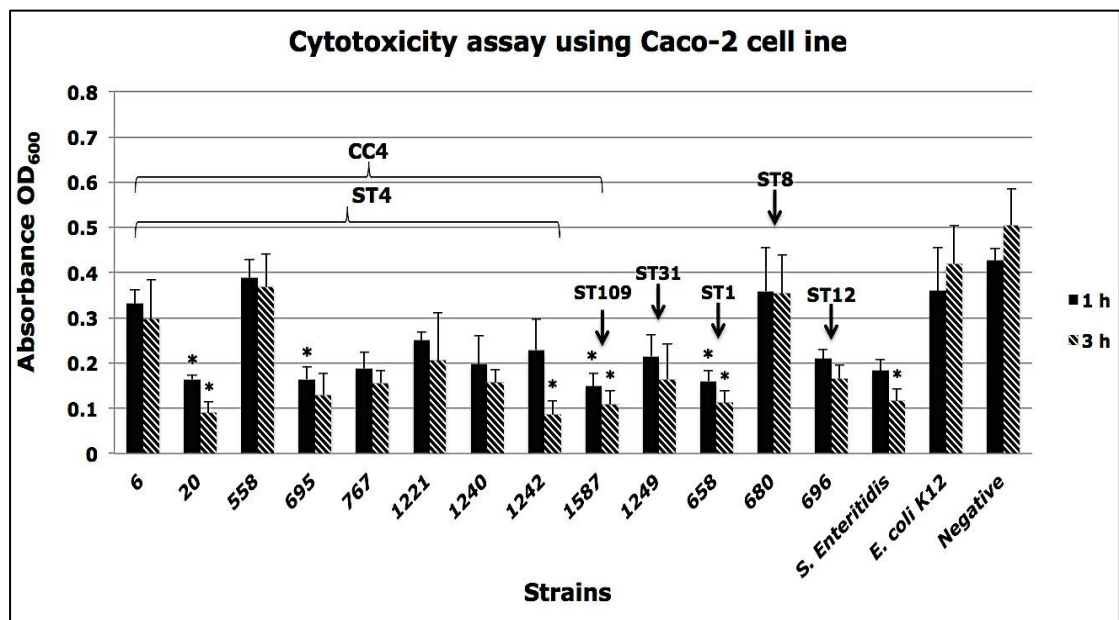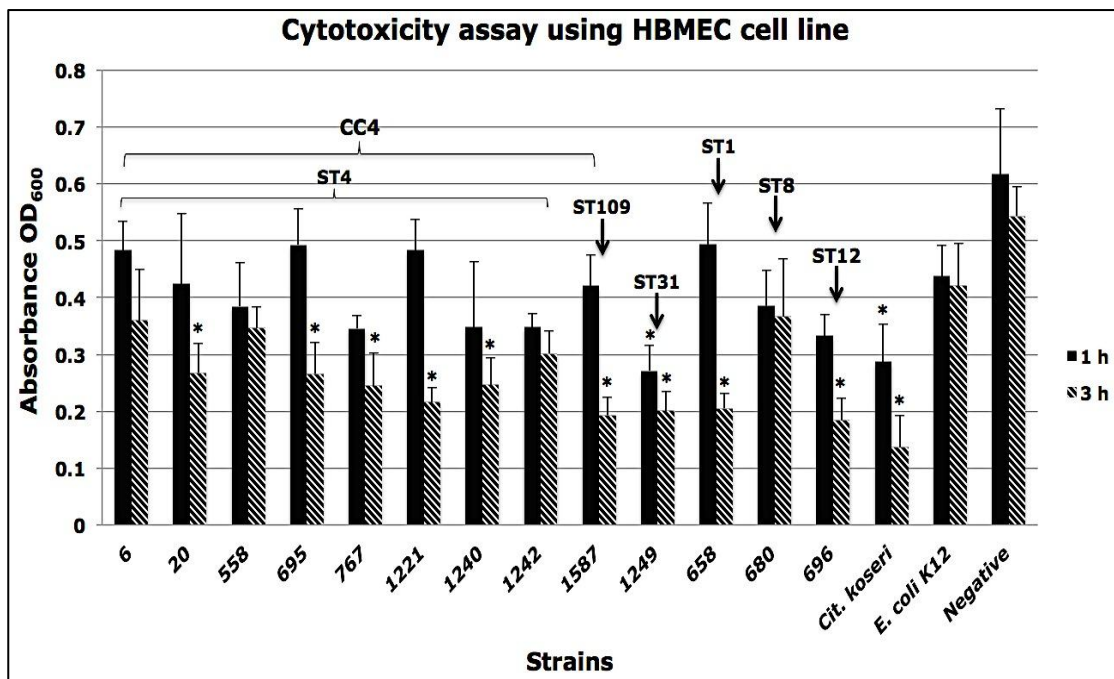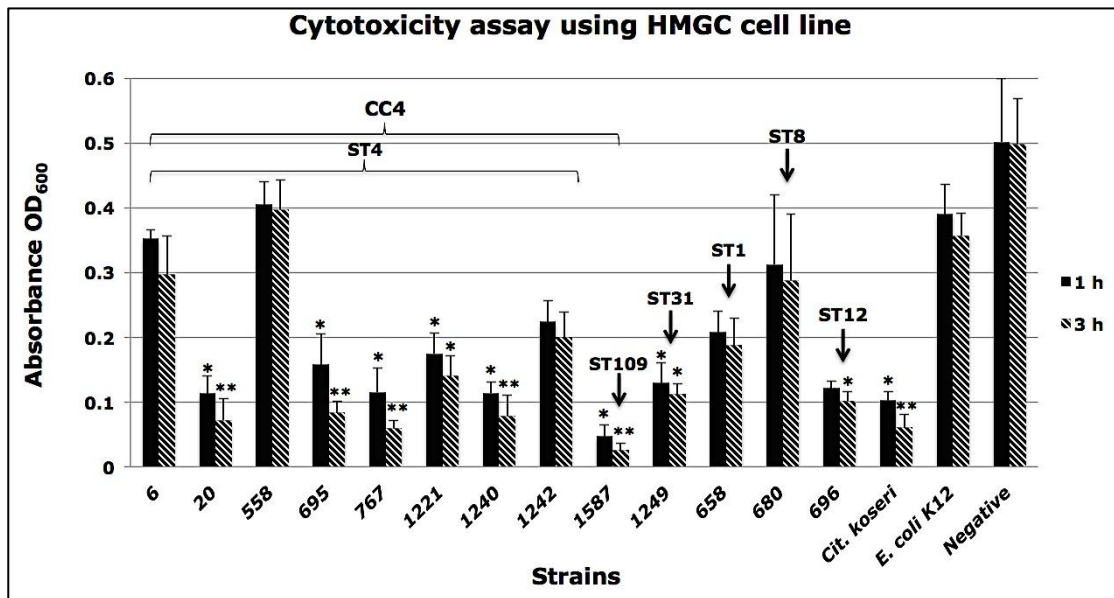
Fig.3.7 Cytotoxicity of *C. sakazakii* strains on HMGC up to 3 hours of incubation. MTT reduction was used to measure the cytotoxicity levels of *C. sakazakii* strains where only the viable HMGC cells are able to reduce MTT to its insoluble purple form formazan, the higher absorbance (OD$_{600}$) the higher in MTT reduction (low toxicity) and vice versa. The negative control used was uninfected cells treated using the same protocol with no bacteria added. The data presented in mean±standard error of mean of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$ and (**$P<0.001$; ANOVA).
ST: Sequence type.

Table.3.3 below summarises the cytotoxicity of *C. sakazakii* strains for different cell lines and the clinical presentation of each strain.

Table.3.3: *C. sakazakii* cytotoxicity and strains clinical presentation.

Strain	ST	Caco-2 cytotoxicity	HBMEC cytotoxicity	HMGC cytotoxicity	Clinical presentation
6	4*	Low	Low	Low	Unknown
20	4*	High	High	High	Unknown
558	4*	Low	Low	Low	Unknown
695	4*	High	High	High	Fatal NEC II
767	4*	High	High	High	Fatal meningitis
1221	4*	Moderate	High	High	Meningitis
1240	4*	Moderate	High	High	Fatal meningitis
1242	4*	High	High	Moderate	Fatal meningitis
1587	109*	High	High	High	Brain damage
1249	31	High	Moderate	High	Fatal infant isolate
658	1	Moderate	High	High	Meningitis
680	8	Low	Low	Low	Unknown
696	12	Moderate	High	High	NEC II

3.3.5.2. *C. sakazakii* attachment and invasion assays

3.3.5.2.1. Attachment

Three cell lines were used in this experiment, Caco-2, HBMEC, and rBCEC4. All strains were able to adhere to these cell lines, however ST4 (part of CC4) strains 20, 695, 721, 767, and 1465 showed highest attachment levels to Caco-2 cell line, while the other ST4 strains displayed moderate levels. Moreover strains 557, 558, and 4 exhibited the lowest attachment levels among CC4 strains. Strain 696 (ST12) showed a high attachment level to Caco-2 cell line whereas the other STs' strains displayed moderate levels of attachment. Strains 1 and 5 had the lowest attachment values among non-CC4 strains (see Fig.3.8). Overall, strains 695 (ST4), 1465 (ST4), 696 (ST12), and *S.* Enteritidis, the positive control, showed the most significant adherence among the strains tested ($P<0.05$).

Fig.3.8 *C. sakazakii* attachment assay using Caco-2 cell line after 1-hour incubation showing the differences in attachment levels among strains. The displayed data are the mean±standard deviation of attachment efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.

Regarding the HBMEC cell line, the majority of CC4 strains showed high attachment levels, while strains 553, 557, 558, and 4 exhibited moderate attachment within CC4. However, strain 6 was the lowest in attachment within the same clonal complex. On the other hand, non-CC4 strains showed moderate attachment levels, except for strain 696 (ST12) that displayed a higher adhesion level. Strain 767 (ST4) and *Cit. koseri* the positive control for the cell line showed the highest attachment levels among all of the test strains ($P<0.05$; see Fig.3.9).

These results indicate that ST4 (CC4) strains generally have the ability to adhere to cell lines more than the non-ST4 strains, nonetheless strain 696 (ST12) also showed a level of adhesion similar to some ST4 strains (695 and 1242). The attachment process indicates their ability to interact and potentially invade these cell lines. However, the invasion assay determined whether they are able to invade or not.

Fig.3.9 *C. sakazakii* attachment assay using HBMEC cell line after 1-hour incubation showing the differences in attachment levels among strains. The displayed data are the mean±standard deviation of attachment efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.

Microscopic examination of *C. sakazakii*-infected Caco-2 and HBMEC cell lines was applied to confirm the adherence ability of the organism (Fig.3.10 and Fig.3.11).
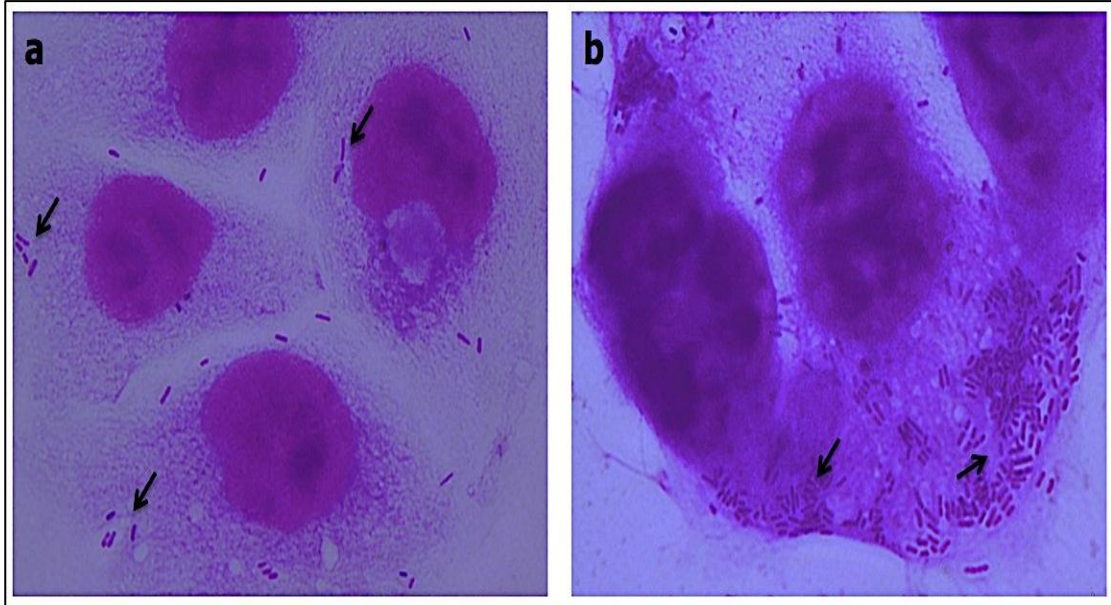
Fig.3.10 *C. sakazakii*-infected Caco-2 cell line for 1 hour, fixed with 100% methanol, stained by 7% Giemsa stain, and examined using oil immersion (100x). The arrows point to bacterial cells attached to Caco-2 cells. **(a)** Non-specific adherence pattern of strain 6 (ST4) that showed moderate adhesion to Caco-2 cell line. **(b)** Localised adherence pattern of strain of strain 696 (ST12) that demonstrated high attachment level.
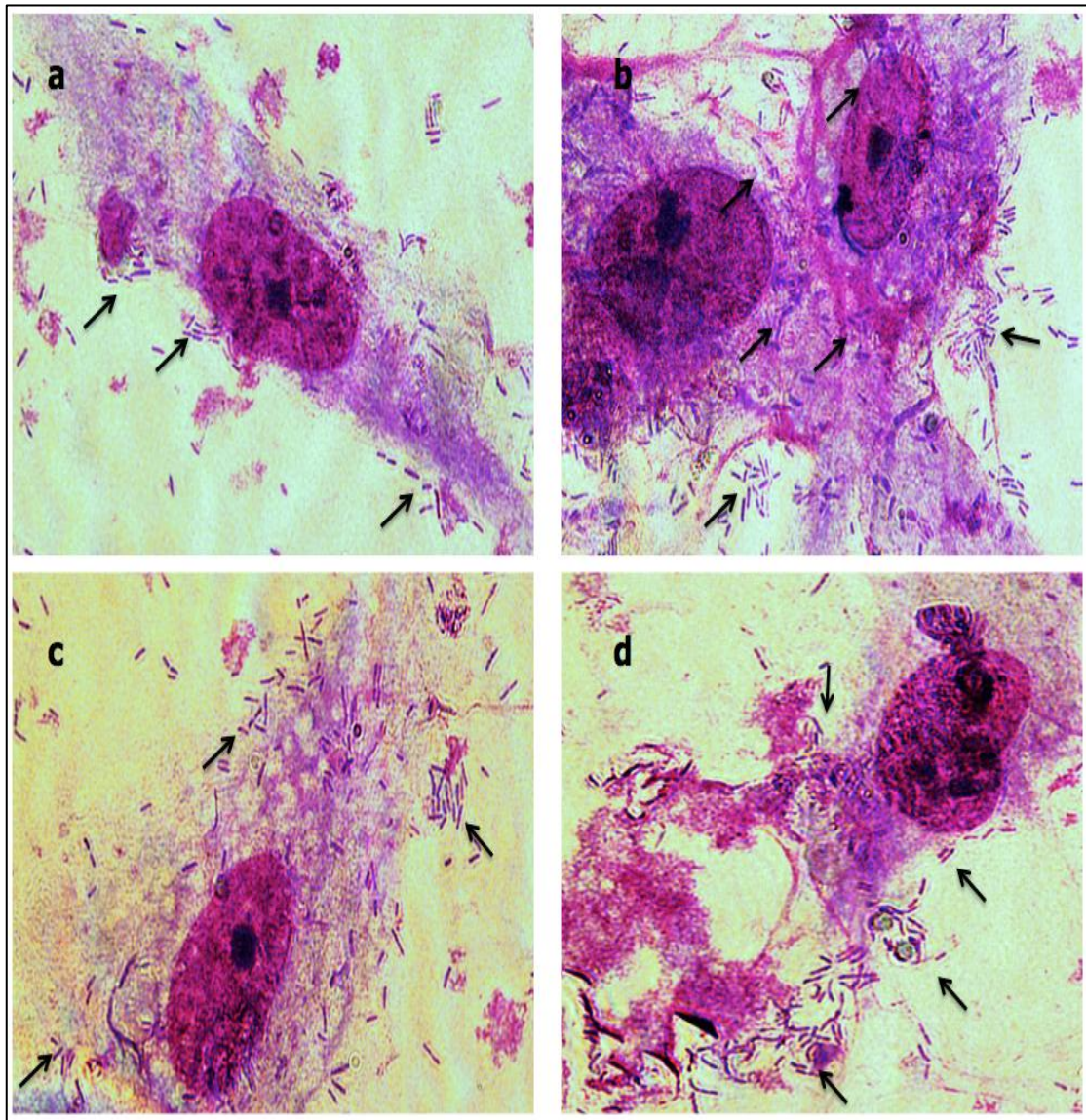
Fig.3.11 *C. sakazakii* strains adhered to HBMEC, which were infected for 1 hour, fixed with 100% methanol, stained by 5% Giemsa stain, and examined using oil immersion (100x). The arrows point to clusters of bacteria attached to the cells. **(a)** Shows localised adherence where the bacterial clusters attached to the cells in certain sites (strain 20). **(b)** Aggregative adherence where the bacteria covered all around the cells (strain 696). **(c)** Non-specific adherence pattern (strain 6). **(d)** Diffused adherence where the bacterial cells surround the cell (strain 695).

With regard to rat brain microvascular endothelial cell line rBCEC4, all the strains were able to attach to these cells (Fig.3.12). CC4 strains 767, 1221, 1587, and strain 696 (ST12) showed highest attachment among the strains tested ($P<0.01$), whereas strains 6 (ST4), 558 (ST4), 680 (ST8), and the *E. coli* K12 (negative control) showed the lowest adherence. Although most ST4 strains showed high ability to attach to this cell line,

strains 6 and 558, however, were low in attachment indicating their low virulence potential (Fig.3.12).
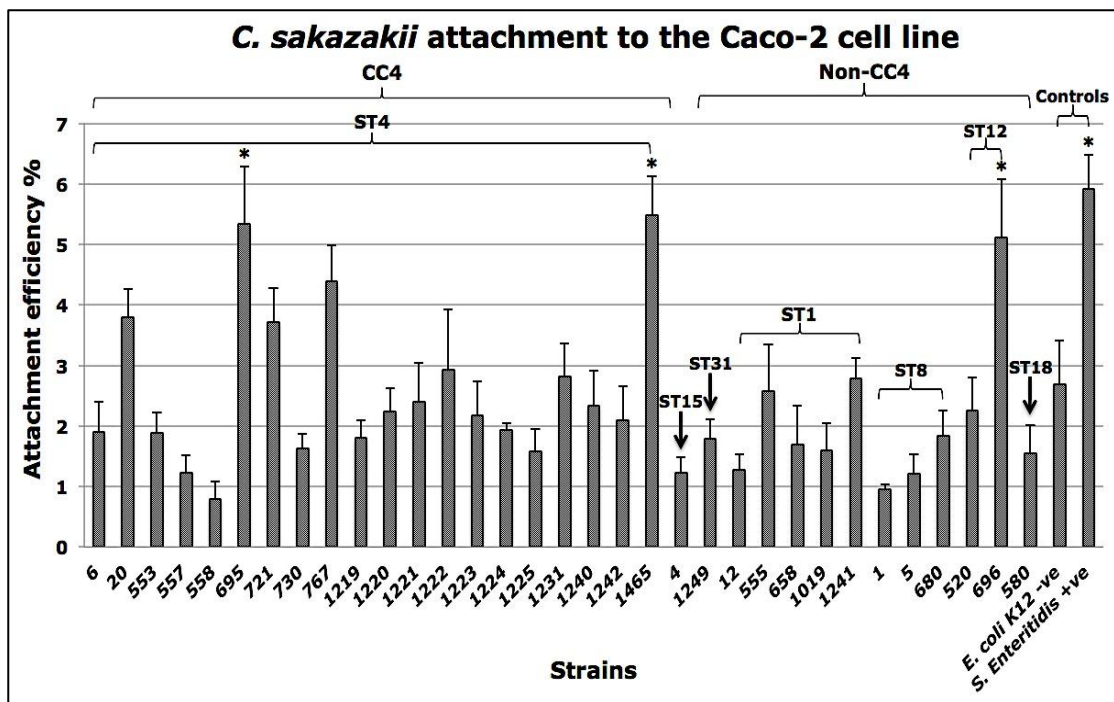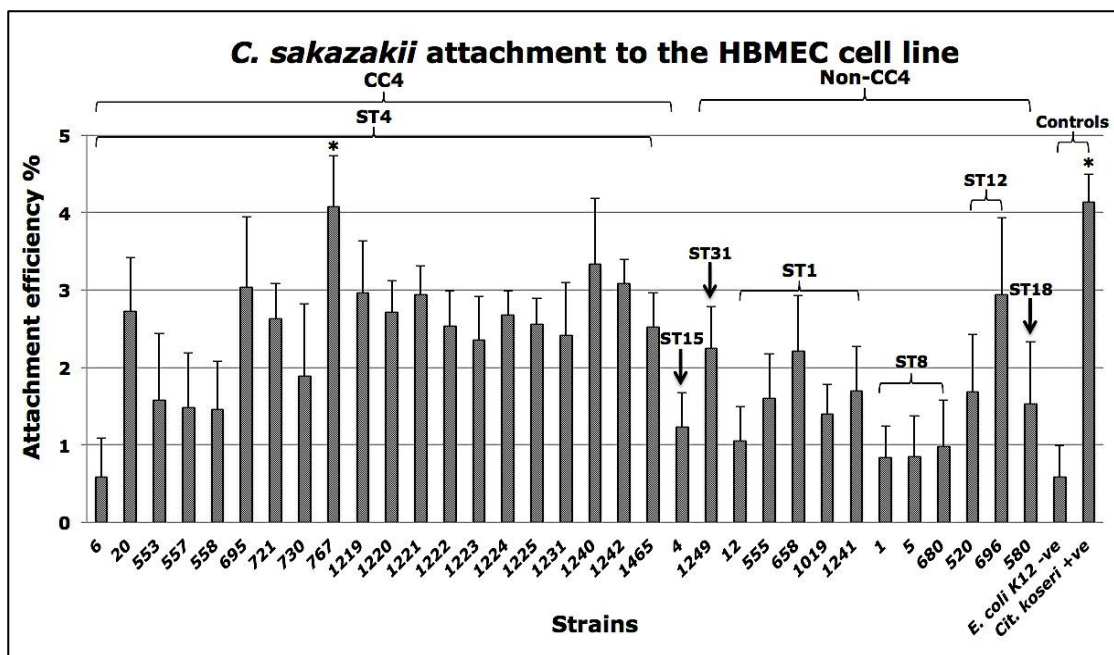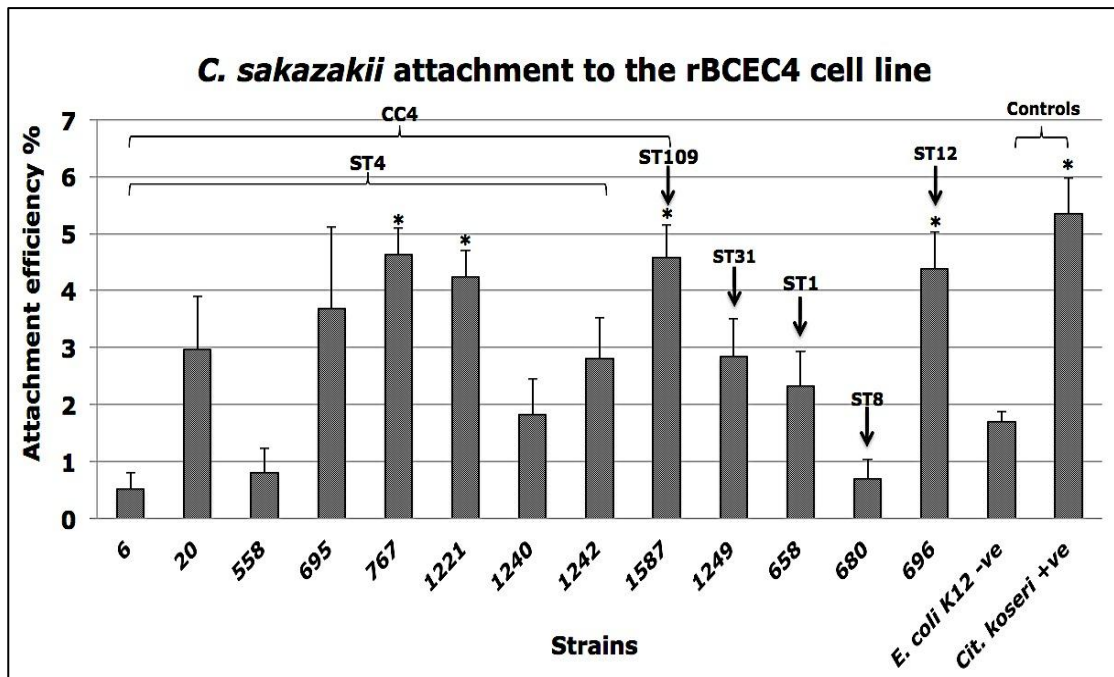

Fig.3.12 *C. sakazakii* attachment assay using rBCEC4 cell line after 1-hour incubation showing the difference in attachment levels among strains. The displayed data are the mean±standard deviation of attachment efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.

3.3.5.2.2. Invasion (gentamicin protection assay)

The invasion assay, using gentamicin protection to kill the extracellular bacteria, was then used to assess the ability of the bacteria to invade Caco-2 and HBMEC cell lines. For the Caco-2 cell line, CC4 strains displayed moderate invasion values, whereas strains 695 ($P<0.05$) and 767 were able to invade at the highest levels compared with the other strains of all STs. Strains 6 (ST4), 730 (ST4), and 4 (ST15) showed the lowest level of invasion within CC4. On the other hand, the non-CC4 strains showed moderate invasion levels, however strains 12 (ST1), 1019 (ST1), 1 (ST8), and 5 (ST8) showed low invasion levels (see Fig.3.13).

Fig.3.13 *C. sakazakii* gentamicin protection assay (GPA) using Caco-2 cell line over 3 hours of incubation showing the differences in invasion levels among strains. The displayed data are the mean±standard deviation of invasion efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.

With regard to the HBMEC cell line, most of CC4 strains showed higher invasion levels than other strains, while strain 730 exhibited a moderate invasion level (Fig.3.14). Strains 553, 557, 558, 1465, and 4 displayed the lowest levels amongst CC4, whereas strain 6 from CC4 was not able to invade the cell line. Non-CC4 generally strains showed low invasion values except for strain 696 (ST12), which showed the highest level among the isolates tested. Strains 1249 (ST31), 658 (ST1), 1241 (ST1), 520 (ST12) and 580 (ST18) were moderate with regard to invasion. *Cit. koseri*, the positive control for the cell line, in addition to strain 767 (ST4) exhibited a significant increase in their ability to invade the HBMEC cell line when compared to other strains ($P<0.01$; see Fig.3.14).
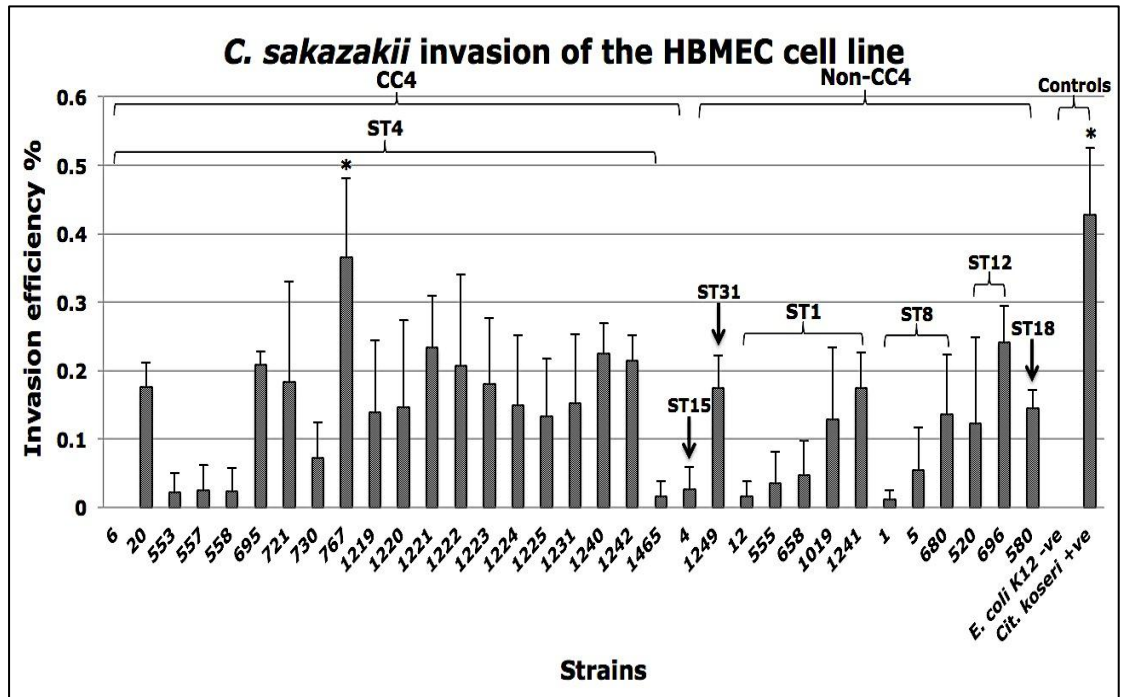
Fig.3.14 *C. sakazakii* gentamicin protection assay **(**GPA) using HBMEC cell line over 3 hours of incubation showing the differences in invasion levels among strains. The displayed data are the mean±standard deviation of invasion efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$; Kruskal-Wallis).

ST: sequence type.
CC4: clonal complex 4.

C. sakazakii invasion of Caco-2 and HBMEC cell lines was examined using microscopy following staining with Giemsa to visualise the invaded bacterial cells. The images are displayed in Fig.3.15 below.
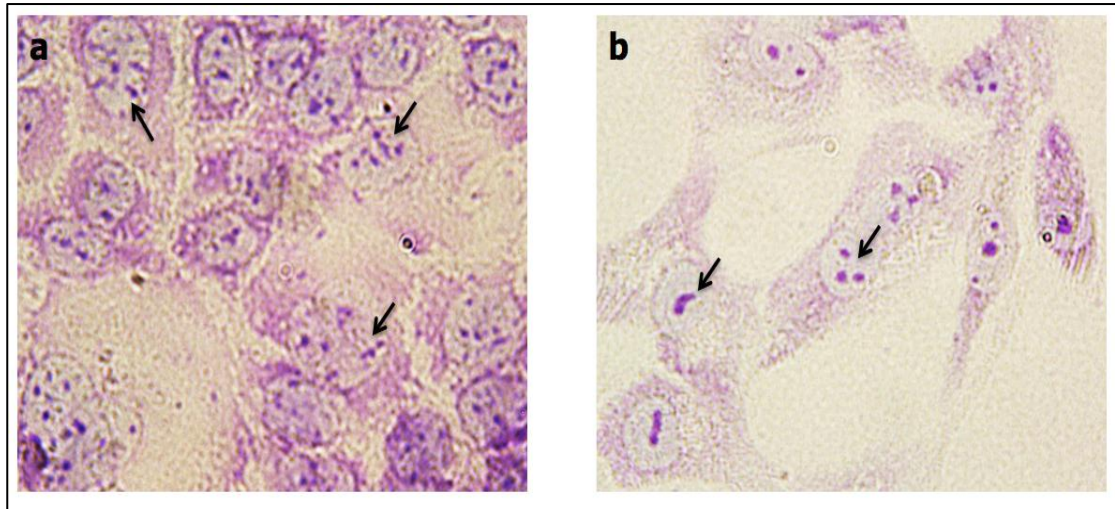
Fig.3.15 *C. sakazakii*-infected Caco-2 and HBMEC cell lines for 3 hours, followed by washing with PBS, addition of gentamicin for 1 hour. Cells were then fixed with 100% methanol, stained with 5% Giemsa stain, and examined using oil immersion (100x). The arrows point to intracellular bacterial cells. **(a)** Intracellular bacterial cells of strain 695 (ST4), which has high invasion ability, inside Caco-2 cells. **(b)** Invasion of *C. sakazakii* strain 767 (ST4) to HBMEC cells that showed high invasion ability for this cell line.

Regarding the invasion of rBCEC4, strain 1221 (CC4), 1587 (CC4), 696 (ST12), and the positive control showed significantly higher invasion levels compared to other isolates tested ($P<0.05$). On the other hand, strains 6 (ST4), 558 (ST4), 658 (ST1), and 680 (ST8) exhibited low invasion levels. With regard to the other strains, they were moderate in their invasion (Fig.3.16). This experiment showed that ST4 strains have the potential for the attachment and invasion more than the other STs similar to their behaviour with the Caco-2 and HBMEC cell lines. However, the invasion levels for rBCEC4 were lower than those of the human cell lines. For strain 696 (ST12), which does not belong to CC4, showed high invasion levels with all cell lines.
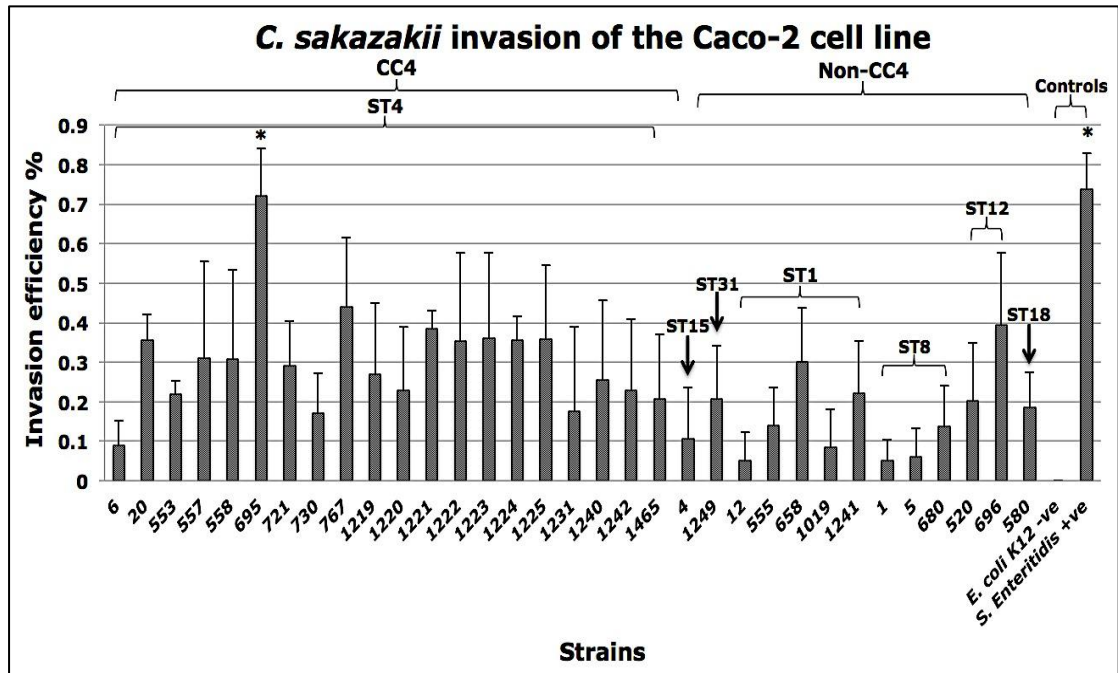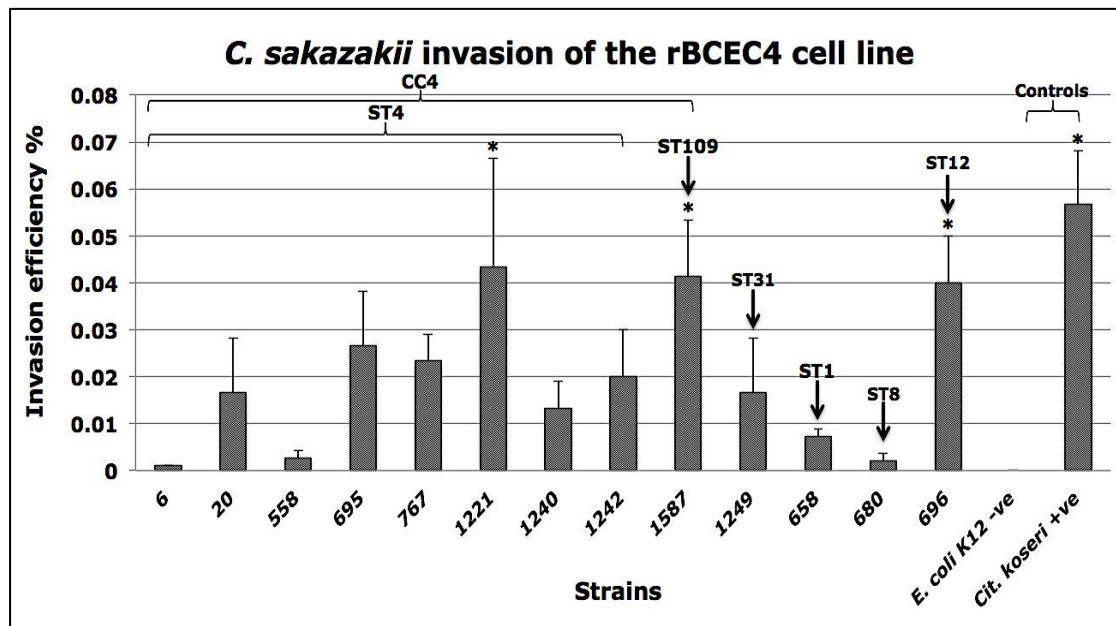
Fig.3.16 *C. sakazakii* gentamicin protection assay GPA using rBCEC4 cell line over 3 hours of incubation showing the difference in invasion levels among strains. The displayed data are the mean±standard deviation of invasion efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.

It was notable that strains 6, 553, 557, 558, and 4 did not show the same attachment and invasion abilities of CC4. Strains 557, 558, and 4 showed low attachment to Caco-2 and HBMEC cell lines. Moreover, these strains in addition to 6 and 553 demonstrated low invasion to the same cell lines. Additionally, it was clear that strain 1465 (ST4) showed high attachment to Caco-2 cell line and low invasion, suggesting that the ability to attach to human cells does not reflect the invasion ability, and this strain could be considered as a low invader.

Strains showing high levels of invasiveness might have the potential to induce more host responses and cause more damage to the infected organs. Therefore, the next experiments used translocation assays to assess the ability of *C. sakazakii* strains to pass through the intact monolayers of the Caco-2 and HBMEC cell lines.

3.3.5.2.3. Invasion-associated genes in *C. sakazakii*

A group of genes were found to have a role in the invasion potential of *Salmonella* and *E. coli* (Farr et al. 1989, Badger et al. 2000, Bessman et al. 2001, Ismail et al. 2003, Peng et al. 2012). These genes differ in their functions and the types of mammalian cells that they mediate the invasion of. The invasion genes that were tested in this project were *apaH*, *ompA*, *ompX*, *ygdP*, and *ppk1*. The gene *apaH* was found to promote invasion to human epithelial cells in *S*. Typhimurium and *E. coli*, while *ompA* is required for Caco-2 cell line invasion. Moreover, *ompA*, *ompX*, *ygdP*, and *ppk1* were associated with HBMEC invasion by *E. coli* K1 (Badger *et al.* 2000, Singamsetty *et al.* 2008, Mohan Nair *et al.* 2009, Kim *et al.* 2010, Peng *et al.* 2012).

C. sakazakii sequenced strains used in this research (*n*=24) were analysed for the presence of *apaH*, *ompA*, *ompX*, *ygdP*, and *ppk1* genes in their genomes (Table.3.2). All strains investigated carried invasion-associated genes, yet showed varied invasion phenotypes suggesting that some of these genes might not be expressed or that different genes are involved in invasion in different strains.

3.3.5.3. Translocation assay
3.3.5.3.1. Translocation assay using the Caco-2 cell line

Translocation is the process whereby the bacterium invades and passes through a polarised monolayer of a cell line. During the first hour of the experiment, the translocation level was low for all strains except for strain 695 (ST4) and *S.* Enteritidis, the positive control, which showed higher translocation than the other strains (Fig.3.17). Different levels of translocation were detected after three hours, however the levels remained low for the majority (94%) of the strains. Strains 695 (ST4), 730 (ST4), and the positive control demonstrated the highest translocation after 3 hours (*P*<0.01). At the fifth hour, most of the CC4 strains translocated to high levels and strain 695 showed highest translocation (*P*<0.01), whereas strains 20 and 553 showed low translocation levels. Strain 6 (ST4) was not able to translocate over the 5 hours of incubation. With regard to non-CC4 strains, they also showed

increasing translocation over time especially strains 658 (ST1), 520 (ST12), and 696 (ST12) that showed moderate translocation after 5h.. In addition, strain 680 (ST8) showed a very low level of translocation , while strains 1019 (ST1), 1 (ST8), 5 (ST8), and *E. coli* K12, the negative control, were not able to translocate (see Fig.3.17).

The transepithelial electrical resistance (TEER) of the Caco-2 monolayers was measured over the period of the assay to monitor the disruption of the tight junctions of the cells. TEER declined in monolayers infected with strains that were able to pass through the intact monolayers, indicating the disruption of the tight junction of the cell line. Moreover, the levels of decline were directly proportional to the capability of the bacterial cells to translocate through this cell line. On the other hand, for the other non-translocating strains the resistance remained steady or only showed a slight decrease (Fig.3.18). A group of *C. sakazakii* strains were able to translocate through Caco-2 monolayers without notable changes in TEER. These strains include CC4 strains 20, 730, 1219, 1221, 1231, 1240, 1242 in addition to 1019 (ST1), 680 (ST8), and 580 (ST18). These strains might use different translocation mechanism other than the disruption of the tight junctions.

As previously mentioned in Section 3.3.5.1, a group of *C. sakazakii* strains were tested for their cytotoxicity for the Caco-2 cell line. It was noted that strains 695 (ST4), 696 (ST12), and *S*. Enteritidis that showed high translocation in addition to strains 1242 (ST4) and 658 (ST1), which were moderate in translocation showed high cytotoxicity to Caco-2. This suggests that cytotoxicity helps the bacterium to abolish the integrity of the cell monolayer by causing cell death leading to the translocation and decreased TEER levels.
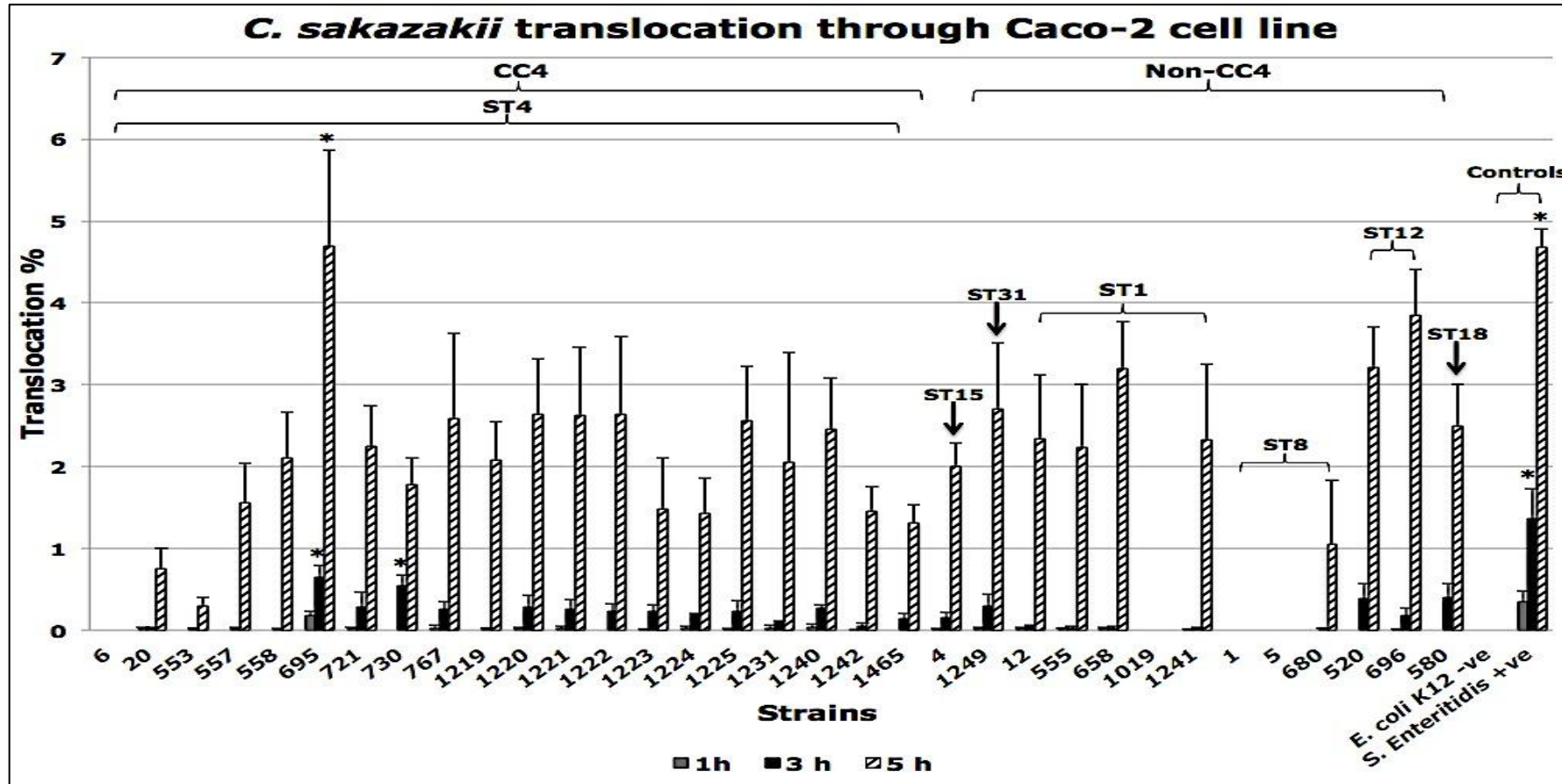
Fig.3.17 Translocation assay using *C. sakazakii*-infected Caco-2 cell line for up to 5 hours of incubation showing the differences in translocation ability among strains. The displayed data are the mean±standard deviation of translocation efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$; Kruskal-Wallis).
ST: sequence type.
CC4: clonal complex 4.
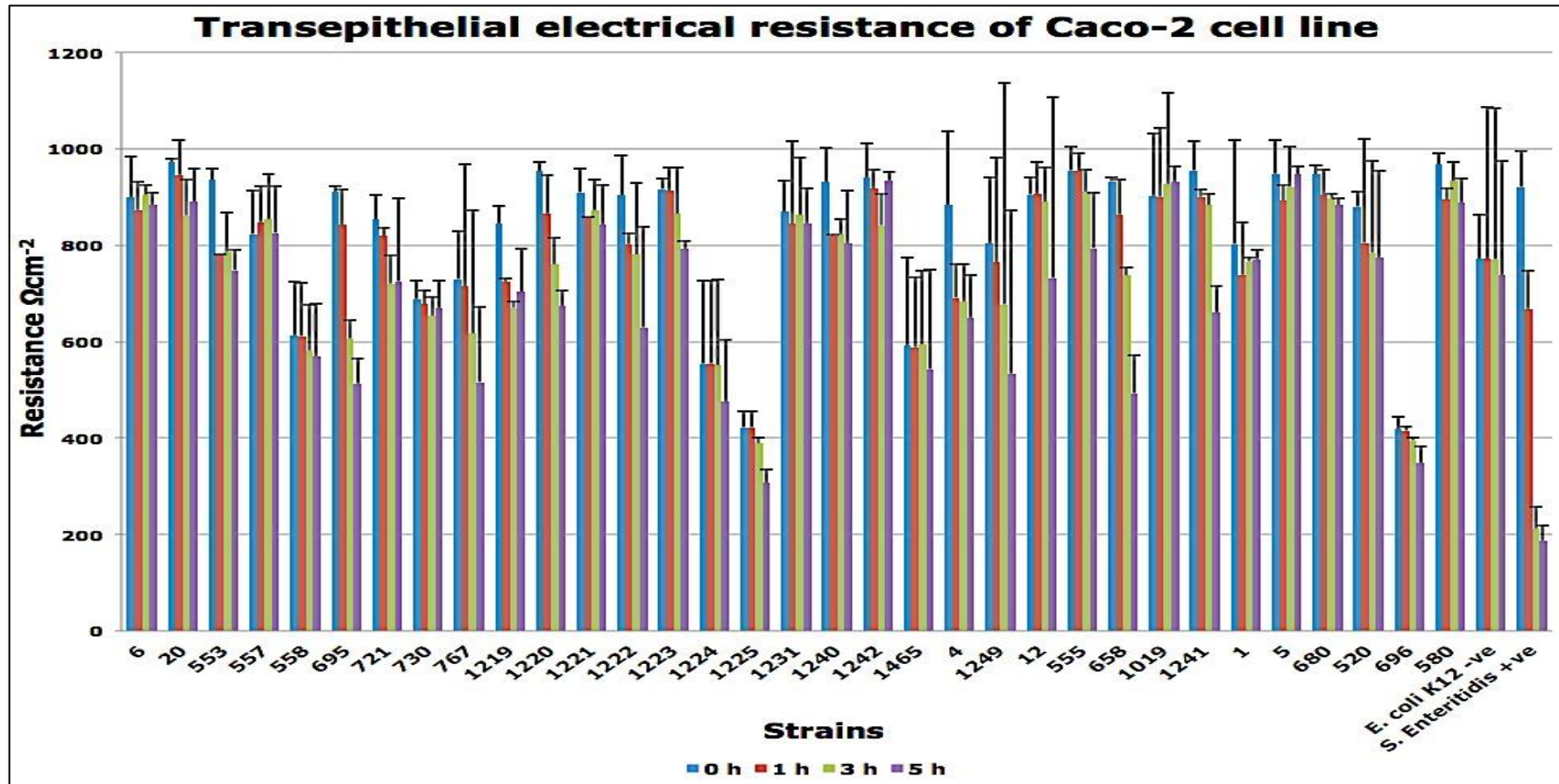
Fig.3.18 Transepithelial electrical resistance (TEER) of Caco-2 monolayers over 5 hours of incubation, showing the changes in electrical resistance. (0 h) is the TEER measurement of uninfected Caco-2 monolayer.

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The effect of *C. sakazakii* strain 695 on Caco-2 cell line was documented using microscopic examination. The images below demonstrate the effect of the organism on the cell line over 5 hours of infection (Fig.3.19). The cells started to die 3 hours following infection and the rounded cells can be seen. After 5 hours, the majority of cells were dead and made floating clumps.

Fig.3.19 Microscopic examination of *C. sakazakii*-infected Caco-2 cell line using strain 695 (ST4). The images show the effect of the infection on the cell line over 5 hours of infection. The arrows point to the dead cells. **(a)** The image shows confluent monolayer of Caco-2 cell line. **(b)** After 3 hour of infection, cells started to die and the changing in shape (rounded cells) is clear. **(c)** 5 hours post infection most of the cells were dead and aggregated into floating clumps.

3.3.5.3.1. Translocation assay using the HBMEC cell line

The HBMEC cell line was used for the second translocation assay. During the initial 4 hours of the incubation period, no strains translocated through the intact monolayer and the cell line exhibited resistance against translocation shown by stable TEER over time (data not shown). After 5 hours of incubation, strains 6 (ST4), 553 (ST4), 558 (ST4), 1465 (ST4), 4 (ST15), 12 (ST1), 1 (ST8), 5 (ST8), and the negative control *E. coli* K12 were not able to pass through the polarised monolayer of the cell line (Fig.3.20). Another group of strains including 557 (ST4), 730 (ST4), 1242 (ST4), 1249 (ST31), 555 (ST1), 658 (ST1), 1019 (ST1), 1241 (ST1), 680 (ST8), 520 (ST12) and 580 (ST18) showed low translocation levels.

Furthermore, CC4 strains 721, 1224, 1225, and 1231 translocated more when compared with the previous group of strains. Moreover, CC4 strains 20, 695, 1219, 1220, 1221, 1222, 1223, and 1240 in addition to strain 696 (ST12), showed significantly elevated translocation among the strains tested ($P<0.01$). Strain 767, and *Cit. koseri*, the positive control, demonstrated the most significant elevation in translocation ($P<0.001$; Fig.3.20).

The electrical resistance of the cell line was mostly steady with slight changes with some strains during the first 4 hours of infection (data not shown). The drops in resistance were noticed after 5 hours of incubation in the wells infected with translocating strains indicating their ability to disrupt the tight junctions and pass through the membrane to the basolateral compartment (Fig.3.21).

The cytotoxic effect of *C. sakazakii* strains was investigated as mentioned previously in Section 3.3.5.1. CC4 strains 20, 695, 767, and 1240 in addition to 696 (ST12), which translocated to a higher level through the HBMEC cell line, showed significantly higher cytotoxicity to this cell line after 3 hours of infection when compared to the negative control of that assay. This correlates with the results of translocation and the decline in TEER, which suggests that cytotoxicity could increase the permeability of this cell line allowing more bacterial cells to pass through. However, this is an apparent contradiction of the results of the previous translocation experiment using Caco-2 cell line, as this cell line was highly susceptible to translocation of the invasive strains.
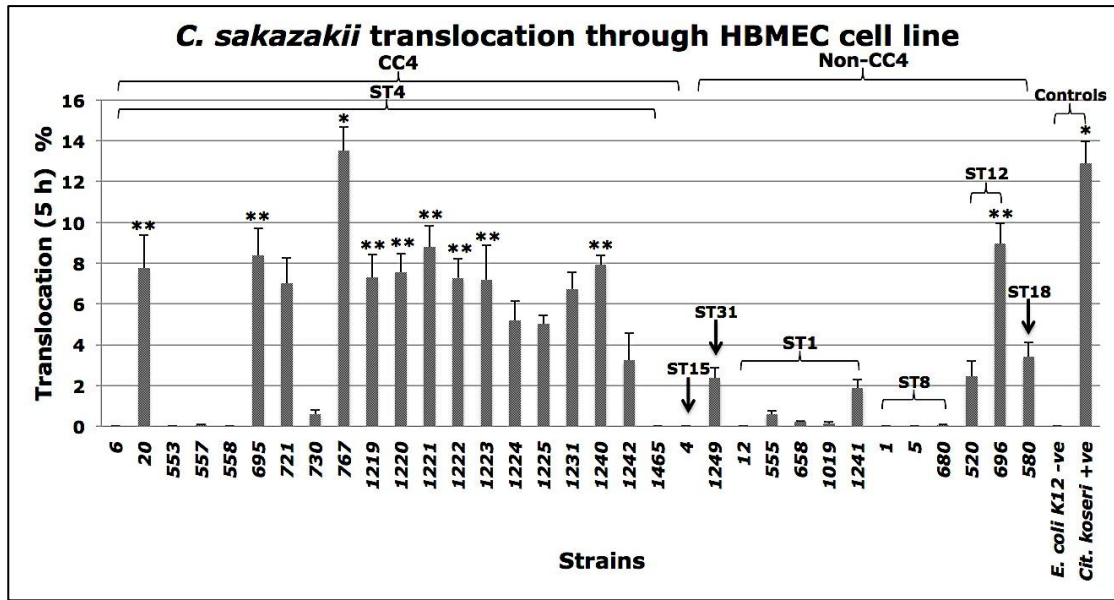
Fig.3.20 *C. sakazakii* translocation assay using the HBMEC cell line after 5 hours of incubation showing the differences in translocation ability among strains. The displayed data are the mean±standard deviation of translocation efficiency as % of the initial inoculum (10^6 cfu/ml) of two independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.001$, ** $P<0.01$; Kruskal-Wallis).
**ST:** sequence type.
CC4: clonal complex 4.
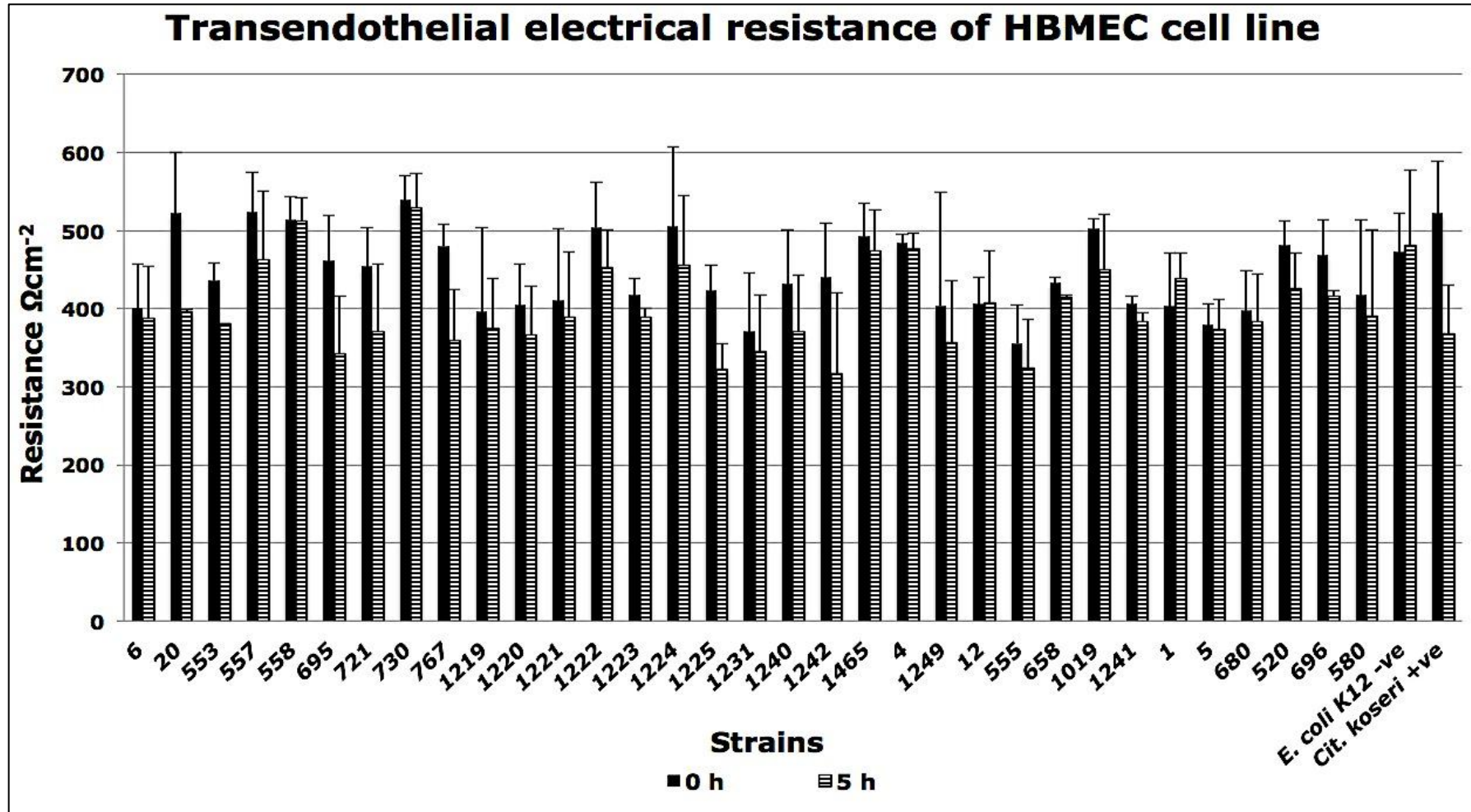
Fig.3.21 Transendothelial electrical resistance (TEER) of the HBMEC cell line over 5 hours of incubation, showing changes in resistance over time.

The effect of *C. sakazakii* strain 1221 on HBMEC cell line was investigated using microscopic examination. The images below show the effect of the bacterium on the cell line over time (Fig.3.22). After 3 hours of infection, the cells were essentially unchanged, while a higher number of dead cells appeared after 5 hours incubation with *C. sakazakii*
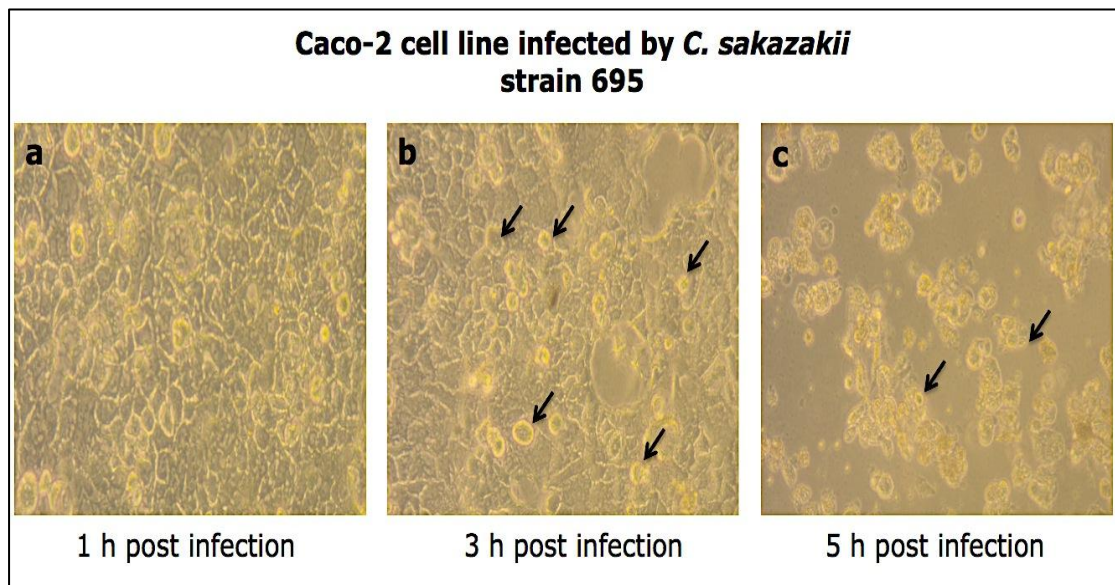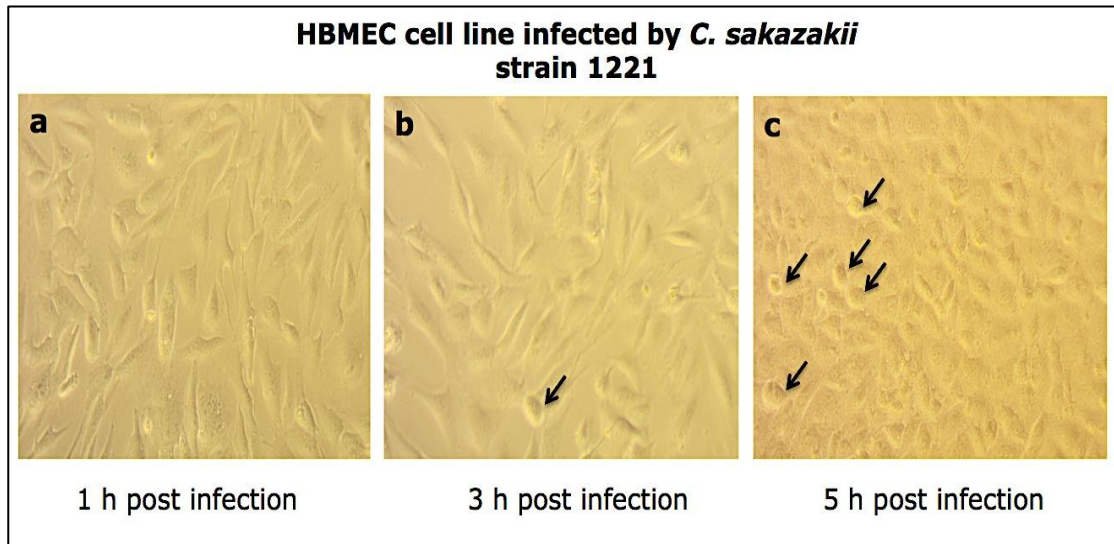

Fig.3.22 Microscopic examination of *C. sakazakii*-infected HBMEC cell line using strain 1221 (ST4). The images show the effect of the infection on the cell line over time. The arrows point to the dead cells. **(a)** HBMEC cells showed normal appearance after 1 hour of infection. **(b)** HBMEC cells showed low susceptibility for bacterial killing 3 hours after infection. **(C)** A number of dead HBMEC cells started to appear 5 hours post infection.

3.3.5.4. Uptake and survival assays inside phagocytic cells

3.3.5.4.1. *C. sakazakii* persistence in human macrophages

Selected *C. sakazakii* strains were examined for the uptake and the survival within human macrophages represented by U937 cell line. These strains represent different sequence types and they vary in their invasion ability of Caco-2 and HBMEC cell lines. Most of the strains persisted in macrophages for 72 hours after the uptake excluding strain 558 (ST4), 680 (ST18) and the negative control *E. coli* K12 that was killed rapidly following uptake (Fig.3.23).

All strains were taken up by macrophages 1-hour post infection. After 6 hours of incubation, most of the strains showed a slight increase in their numbers except for strains 6 (ST4), 558 (ST4), 680 (ST18), and the negative control that displayed a slight decrease.

After 24 hours of incubation, ST4 strains 20, 695, 767, and 1221 in addition to strains 658 (ST1), 696 (ST12), and the positive control *Cit. koseri* were able to multiply significantly compared to other strains (*P*<0.05). On the other hand, strains 6 (ST4), 558 (ST4), and 680 (ST18) exhibited a drop in their numbers (>50%), while strains 695 (CC4), 1240 (CC4), 1242 (CC4), and 1587 (CC4) were able to multiply and demonstrated moderate survival rates. Strain 1249 (ST31) showed only a slight change in viability on incubation with U937 cells over the first 24 hours of incubation.

All strains displayed slight declines in their intracellular survival after 48 hours of incubation, whereas strains 558 (ST4), 680 (ST18), and the negative control declined significantly (*P*<0.05) losing more than 70% of their numbers. The survival rate after 72 hours post infection was high for strains 695 (ST4), 767 (ST4), 1221 (ST4), and the positive control, whereas the other strains declined significantly (*P*<0.001), specifically strains 6 (ST4), 558 (ST4), 680 (ST18), and the negative control (Fig.3.23).
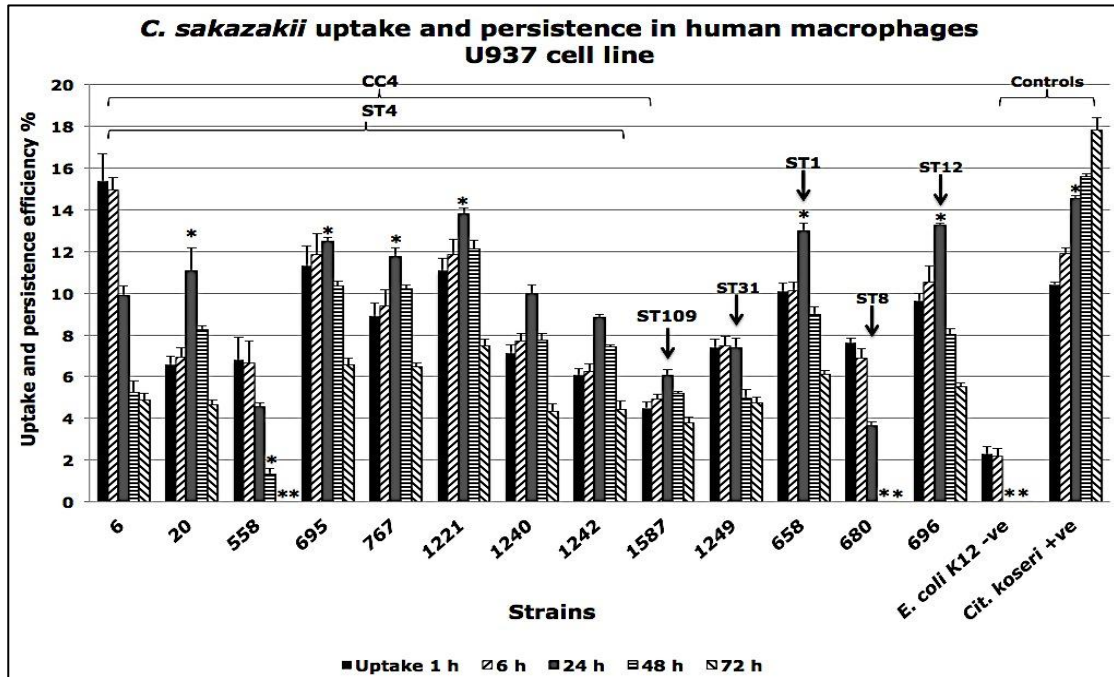
Fig.3.23 *C. sakazakii* uptake and persistence assay using U937 cell line over 72 hours of incubation showing the differences in survival among strains. The displayed data are the mean±SEM for uptake and persistence efficiency as % of the initial inoculum (10^5 cfu/ml) of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$, **$P<0.001$; ANOVA).
**ST:** sequence type.
CC4: clonal comlex 4.

3.3.5.4.2. *C. sakazakii* persistence in human microglial cells

C. sakazakii is linked to fatal meningitis cases, and therefore it is important to show its ability to resist phagocytosis and withstand killing inside the brain. This experiment was conducted to assess the ability of *C. sakazakii* to survive within microglial cells, the brain resident macrophages, and multiply intracellularly.

All strains were taken up by microglia 1-hour post infection (Fig.3.25). Twenty-four hours post infection, most of the strains showed decreased survival levels especially strains 6 (ST4), 558 (ST4), 680 (ST8), 696 (ST12), and the negative control *E. coli* K12 that declined markedly showing more than 50% decrease in their numbers. On the other hand, CC4 strains 20, 695, 767, 1221, 1240, and 1587 in addition to strain 1249 (ST31) and *Cit. koseri*, the positive control, demonstrated significant survival and multiplication levels (*$P<0.01$) scoring more than 10%

increase in their levels. All strains showed decreased persistence levels (up to 40%) 48 hours following infection except for strain 767 (ST4) and the positive control, which showed significantly higher persistence levels ($P<0.05$). After 72 hours of incubation, the strains continued to show a decrease in their intracellular numbers (>50%), however the survival levels of strain 20 (ST4) and the positive control remained significantly higher ($P<0.01$; Fig.3.25).

About 77% of the strains were able to survive within microglia for 72 hours, which indicates their virulence potency. Fifty six percent of CC4 strains, some of which were linked to meningitis cases, have the highest ability to survive and multiply inside these phagocytic cells. This mechanism is important to evade the immune response and these cells could potentially act an incubator *in vivo* to produce more bacterial cells and causing more damage to the brain during infection. There was a correlation between the survival ability and the cytotoxic effect of some strains (Fig.3.7), as the higher in cytotoxicity the higher in survival. Fig.3.24 below demonstrates the uptake of strain 1240 (ST4) by microglial cells. A summary of the previous results in this section is provided in the tables below (Table.3.4-5).
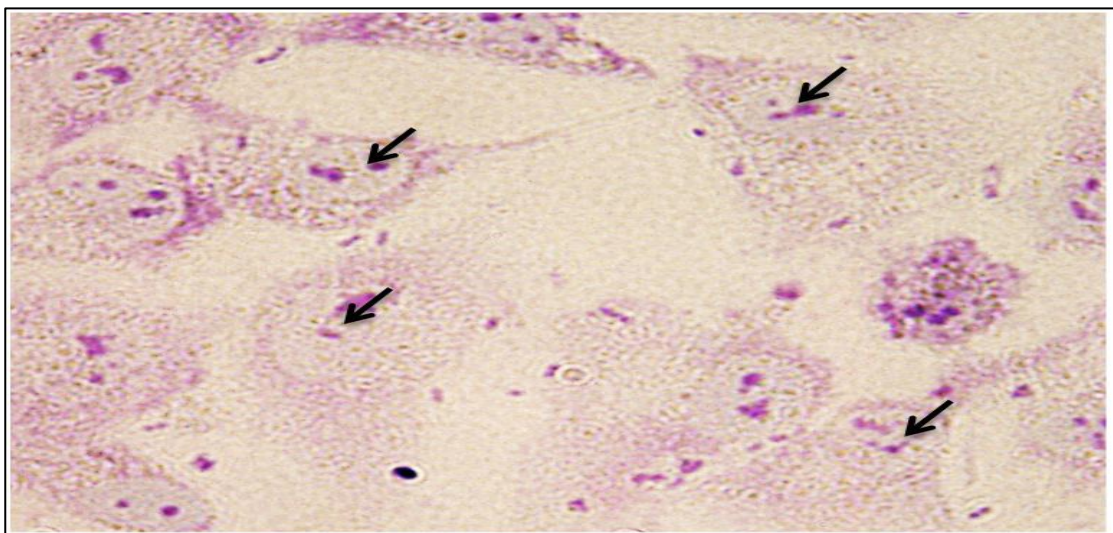
Fig.3.24 Uptake of *C. sakazakii* cells by HMGC cells. The cells were infected with strain 1240 (ST4) for 1 hour followed by addition of gentamicin for 1 hour, fixation with 100% methanol and staining with 5% Giemsa, and examined using oil immersion (100x). Arrows point to engulfed bacterial cells.
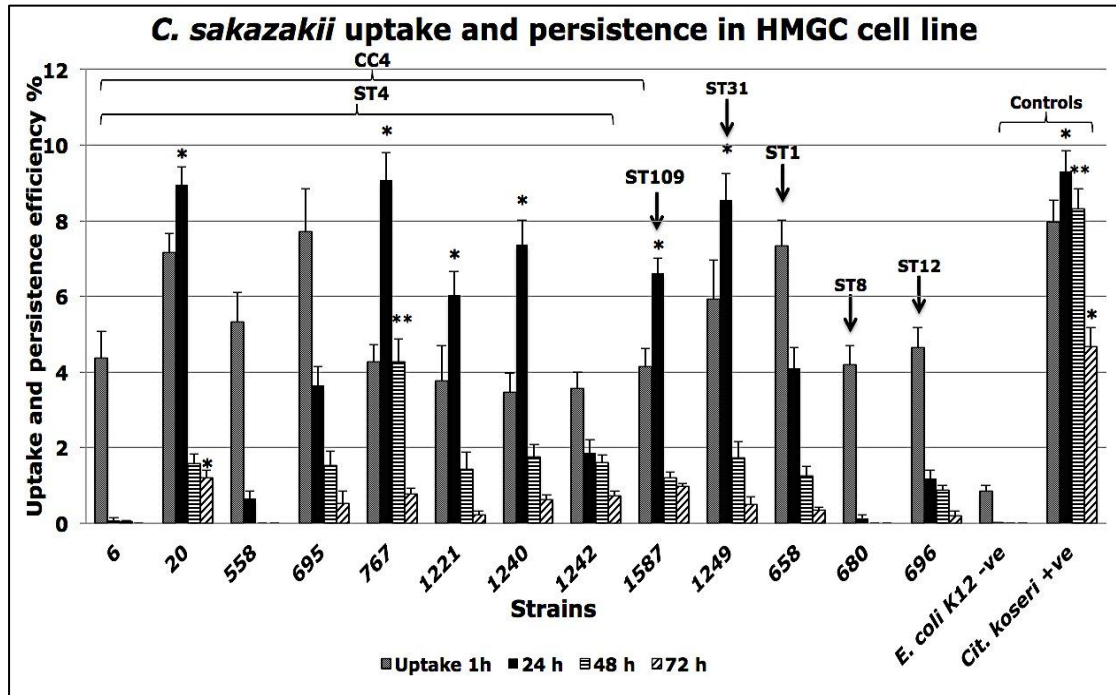
Fig.3.25 *C. sakazakii* uptake and persistence assay using HMGC cell line over 72 hours of incubation showing the differences in survival among strains. The displayed data are the mean±SEM for uptake and persistence efficiency as % of the initial inoculum (10^5 cfu/ml) of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.001$, **$P<0.05$; ANOVA).

ST: sequence type.
CC4: clonal comlex 4.

The effect of *C. sakazakii* strain 767 on HMGC cell line was investigated using microscopic examination. Fig.3.26 shows the effect at three different time points (Fig.3.26). The dead rounded microglial cells started to appear 3 hours post infection, and were aggregated in clumps of dead cells after 5 hours.
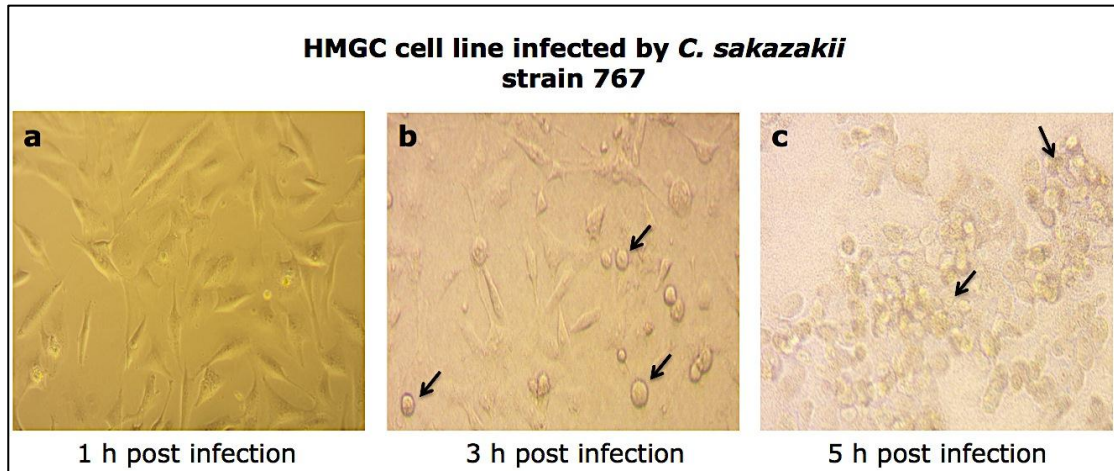
Fig.3.26 Microscopic examination of *C. sakazakii*-infected HMGC cell line using strain 767 (ST4). The images show the effect of the infection on the cell line over 5 hours of infection. The arrows point to the dead cells. **(a)** HBMEC cells showed normal adherent cells after 1 hour of infection. **(b)** A number of detached dead cells started to appear after 3 hours post infection. **(C)** A group of dead HBMEC cells aggregated into clumps 5 hours after infection.

3.3.5.4.1. Phagocytosis resistance-associated genes

It was shown that the PhoP/PhoQ regulatory system of *S.* Typhimurium and *Y. pestis* and their regulated genes including *pmrABE* and *mgtB* play a critical role in phagocytosis survival (Ernst *et al.* 1999, Grabenstein *et al.* 2006). Moreover, *gsrA* of *Y. enterocolitica* was shown to provide protection against oxidative stress killing by macrophages (Yamamoto *et al.* 1996). Additionally, the product of the *sodA* gene, superoxide dismutase (SOD), protects bacteria against phagocytosis as the first contact occurs at the surface of the phagocyte. This is by the conversion of the reactive superoxide radicals into molecular oxygen (Beaman and Beaman 1984, Cox *et al.* 2003). This reaction is important to *C. sakazakii* strains to avoid effects of phagocytosis by macrophages and microglia.

All sequenced strains used in this project (*n*=24) have *phoP*, *phoQ*, *pmrABE*, and *sodA* genes in their genomes (Table.3.2) Moreover, 87% of strains (*n*=24) encode the *mgtB* gene, while ST4 strains 6 and 557 in addition to strain 520 (ST12) were negative for this gene. Strain 6 (ST4) that lacks the *mgtB* gene was not able to survive within macrophages and microglial cells (Fig.3.23 and Fig.3.25). On the other hand, strains 558 (ST4) and 680 (ST8) that are positive for the *mgtB* gene also displayed the same behaviour as strain 6 (ST4) in the survival ability inside macrophages and microglial cells. Although strains 658 (ST1) and 696 (ST12) were positive for all genes investigated, they were able to multiply within macrophages but not in microglial cells (Fig.3.23 and Fig.3.25). All other strains were positive for the tested phagocytosis-resistance related genes and showed the ability to persist and multiply within macrophages and microglial cells (Fig.3.23 and Fig.3.25).

Table.3.4-5 summarise the results obtained in this chapter.

Table.3.3: Results summary of motility, iron siderophore production, serum resistance, rBCEC4 attachment and invasion, and the source and clinical presentation of strains.

Strains	ST	Source	Clinical presentation	Motility[1]	Iron siderophores production[2]	Serum resistance[3]	rBECE4 attachment[4]	rBECE4 invasion[5]
695[a]	4	Clinical - Trachea	Fatal NEC II	Motile	+	Resistant	High	Moderate
767[a]	4	Clinical - Trachea	Fatal meningitis	Motile	+	Resistant	High	Moderate
696[a]	12	Clinical - Faeces	NEC II	Motile	+	Resistant	High	High
1587	109	Clinical-CSF	Brain damage	Motile	+	Resistant	High	High
20	4	Clinical	Unknown	Motile	+	Resistant	High	Low
1221	4	Clinical - CSF	Meningitis	Motile	+	Resistant	High	High
1240	4	Clinical - CSF	Fatal meningitis	Motile	+	Resistant	Low	Low
1249	31	Clinical	Fatal infant isolate	Motile	+	Resistant	High	Low
1242	4	Clinical - Brain	Fatal meningitis	Motile	+	Resistant	High	Low
658*	1	Non-infant formula	Meningitis	Motile	+	Resistant	Moderate	Low
680	8	Clinical - CSF	Unknown	Non-motile	+	Sensitive	Low	Low
558	4	Clinical	Unknown	Motile	+	Resistant	Low	Low
6[c]	4	Clinical	Unknown	Motile	-	Sensitive	Low	Low
553	4	Clinical	Unknown	Motile	+	ND	ND	ND
557	4	Clinical	Unknown	Motile	+	ND	ND	ND
721	4	Clinical - CSF	Unknown	Motile	+	ND	ND	ND
730[a]	4	Clinical	NEC I	Motile	+	ND	ND	ND
1220	4	Clinical - CSF	Brain abscess, not fatal	Motile	+	ND	ND	ND
1222	4	Clinical - Blood	Fever	Motile	+	ND	ND	ND
1223	4	Clinical - Blood	Unknown	Non-motile	+	ND	ND	ND
1224	4	Clinical - Blood	Fever	Non-motile	+	ND	ND	ND
1225	4	Clinical - Blood	Fatal meningitis	Motile	+	ND	ND	ND
1231	4	Clinical - Faeces	Meningitis	Motile	+	ND	ND	ND
1465	4	Infant formula	Unknown	Motile	+	ND	ND	ND
4	15[b]	Clinical	Unknown	Motile	+	ND	ND	ND
12	1	Clinical - Faeces	Unknown	Motile	+	ND	ND	ND
555	1	Clinical	Unknown	Motile	+	ND	ND	ND
1019**	1	Clinical - CSF	Meningitis	Motile	+	ND	ND	ND
1241	1	Clinical - Blood	Unknown	Motile	+	ND	ND	ND
1	8	Clinical - Throat	Unknown	Motile	+	ND	ND	ND
5	8	Clinical	Unknown	Motile	+	ND	ND	ND
520[c]	12	Clinical	Unknown	Motile	-	ND	ND	ND
580	18	Clinical	Unknown	Motile	+	ND	ND	ND

ST: sequence type. **+:** able to produce. **-:** Not able to produce. **ND:** not done. **CSF:** Cerebrospinal fluid. **NEC:** Necrotising enterocolitis.
rBCEC4: rat brain endothelial cells. ***:** BAA-894 the first *C. sakazakii* strain sequenced (Kucerova *et al.* 2010). ****:** Alternative culture collection code HPB-3290.
[a]: French outbreak strains (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2008).
[b]: Part of clonal complex 4, which contains STs 4, 15, 97, 107, 108, and 109 (Forsythe *et al.* 2014). [c]: Plasmid-less strains.
[1]: Fig.3.1. [2]: Fig.3.2. [3]: Fig.3.3. [4]: Fig.3.12. [5]: Fig.3.16.

Table.3.4: Results summary of cytotoxicity, attachment, invasion, translocation, and phagocytosis survival.

Strains	ST	Caco-2 cytotoxicity[1]	Caco-2 attachment[2]	Caco-2 invasion[3]	Caco-2 translocation[4]	HBMEC cytotoxicity[5]	HBMEC attachment[6]	HBMEC invasion[7]	HBMEC translocation[8]	U937 survival[9]	HMGC cytotoxicity[10]	HMGC survival[11]
695[a]	4	High	High	High	High	High	High	Moderate	High	P/M	High	P/K
767[a]	4	High	High	High	Moderate	High	High	High	High	P/M	High	P/M
696[a]	12	Moderate	High	Moderate	High	High	High	High	High	P/M	High	P/K
1587[c]	109	High	High	High	High	High	High	High	ND	P/M	High	P/M
20	4	High	High	Moderate	Low	High	High	Moderate	High	P/M	High	P/M
1221	4	Moderate	Moderate	Moderate	Moderate	High	High	High	High	P/M	High	P/M
1240	4	Moderate	Moderate	Moderate	Moderate	High	High	High	High	P/M	High	P/M
1249	31	High	Moderate	Moderate	Moderate	Moderate	High	Moderate	Low	P/M	High	P/M
1242	4	High	Moderate	Moderate	Low	High	High	High	Low	P/M	High	P/K
658[*]	1	Moderate	Moderate	Low	High	High	High	Low	Low	P/M	Moderate	P/K
680	8	Low	Moderate	Moderate	Low	Low	Low	Low	Low	P/K	Low	P/K
558	4	Low	Low	Moderate	Moderate	Low	Moderate	Low	No	P/K	Low	P/K
6	4	Low	Moderate	Low	No	Low	Low	No	No	P/K	Low	P/K
553	4	ND	Low	Moderate	Low	ND	Moderate	Low	No	ND	ND	ND
557	4	ND	Low	Moderate	Low	ND	Moderate	Low	Low	ND	ND	ND
721	4	ND	High	Low	Moderate	ND	High	Moderate	Moderate	ND	ND	ND
730[a]	4	ND	Moderate	Moderate	Low	ND	Moderate	Low	Low	ND	ND	ND
1219	4	ND	Moderate	Moderate	Moderate	ND	High	Moderate	High	ND	ND	ND
1220	4	ND	Moderate	Moderate	Moderate	ND	High	Moderate	High	ND	ND	ND
1222	4	ND	Moderate	Moderate	Moderate	ND	High	Moderate	High	ND	ND	ND
1223	4	ND	Moderate	Moderate	Low	ND	High	Moderate	High	ND	ND	ND
1224	4	ND	Moderate	Moderate	Low	ND	High	Moderate	Moderate	ND	ND	ND
1225	4	ND	Moderate	Moderate	Moderate	ND	High	Moderate	Moderate	ND	ND	ND
1231	4	ND	Moderate	Moderate	Moderate	ND	High	Moderate	Moderate	ND	ND	ND
1465	4	ND	High	Low	Low	ND	High	Low	No	ND	ND	ND
4	15[b]	ND	Moderate	Moderate	Moderate	ND	Moderate	Low	No	ND	ND	ND
12	1	ND	Moderate	Moderate	Moderate	ND	Moderate	Low	No	ND	ND	ND
555	1	ND	Moderate	Moderate	Moderate	ND	Moderate	Low	Low	ND	ND	ND
1019[**]	1	ND	Moderate	Low	No	ND	Moderate	Low	Low	ND	ND	ND
1241	1	ND	Moderate	Moderate	Moderate	ND	Moderate	Moderate	Low	ND	ND	ND
1	8	ND	Low	Low	No	ND	Low	Low	No	ND	ND	ND
5	8	ND	Low	Low	No	ND	Low	Low	No	ND	ND	ND
520	12	ND	Moderate	Moderate	High	ND	Moderate	Low	Low	ND	ND	ND
580	18	ND	Moderate	Moderate	Moderate	ND	Low	Low	Low	ND	ND	ND

ST: sequence type. **ND:** not done. **P/M:** persisted/multiplied. **P/K:** persisted/killed. **HBMEC:** human brain microvascular endothelial cells cell line.
U937: human macrophage cell line. **HMGC:** human microglial cell line.
*: BAA-894 the first *C. sakazakii* strain sequenced (Kucerova *et al.* 2010). ****:** Alternative culture collection code HPB-3290.
[a]: French outbreak strains (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2008). [b]: Part of clonal complex 4, which contains STs 4, 15, 97, 107, 108, and 109 (Forsythe *et al.* 2014). [c]: The attachment, invasion, and translocation through Caco-2 cell line in addition to attachment and invasion of HBMEC cell line experiments were performed with another group of strains and the results can be found in Appendix.1.
[1]: Fig.3.5. [2]: Fig.3.8. [3]: Fig.3.13. [4]: Fig.3.17. [5]: Fig.3.6. [6]: Fig.3.9. [7]: Fig.3.14. [8]: Fig.3.20. [9]: Fig.3.23. [10]: Fig.3.7. [11]: Fig.3.25.

3.4. Discussion

The *Cronobacter* genus contains 7 different species including *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis* (Joseph *et al.* 2012a, Brady *et al.* 2013, Holý and Forsythe 2014, Jackson *et al.* 2014). *C. sakazakii* isolates represent 72.1% of the total *Cronobacter* genus isolates, and the organism was linked to several severe and fatal NEC and meningitis cases around the world. Moreover, it contains different clonal complexes (CC) including CC4 that contains sequence type 4 (ST4) and was the most frequent clonal complex isolated from CSF and meningitis cases (Forsythe *et al.* 2014).

The motility of the bacterium is important for its adherence and passage through the invaded tissues. Moreover, it is important for the organism to acquire nutrients such as iron that allow its continuous growth and multiplication and could help in its virulence. Evading host response, such as serum resistance, is an essential strategy for the survival of *C. sakazakii* strains inside the body. All the previous factors are crucial to the organism to maintain cellular activity, cause cytotoxicity, and invade and translocate through host tissues. The previously mentioned bacterial effects were examined in this chapter to assess the virulence potential of *C. sakazakii* strains and clarify their behaviours.

3.4.1. Motility

Flagella are the bacterial organelles that are responsible for motility, aiding adherence, and helping in bacterial cell translocation. It was reported by Cruz-Córdova *et al.* (2012) that the flagella of *C. sakazakii* play an important role in triggering the host immune response and the production of cytokines such as IL-8 and TNF-α. Furthermore, flagella help in the adhesion to the mammalian cells such as Caco-2 (Hartmann *et al.* 2010). The study of Cruz-Córdova *et al.* (2012) used the strains *C. sakazakii* ATCC BAA-894 (ST1) and *C. sakazakii* ATCC 29004 (ST4) in their experiments. It showed the ability of the flagellated strains to adhere and invade the mammalian cells and trigger cytokine secretion, which was

mentioned previously. According to Hartmann *et al.* (2010), the absence of flagella in *Cronobacter* mutants led to a significant reduction in adhesion capacity to the Caco-2 cell line. This suggests that the presence of flagella, as one of the virulence factors, have an essential role in pathogenesis.

Most of *C. sakazakii* strains that were used in this project were motile except for strains 1223 (ST4), 1224 (ST4), and 680 (ST8). The motility suggests the presence of flagella in the motile strains that gives them the advantage to move and aid adherence to abiotic surfaces and the eukaryotic cells. It was found that about 96% of sequenced strains have all the *fli* genes (Table.3.1) that are responsible for flagella expression and movement according to previous studies (Kucerova *et al.* 2010, Joseph *et al.* 2012b). Strain 520 (ST12) was the only motile strain that was negative for *fliN*, *fliO*, *fliP*, *fliQ*, and *fliR* genes indicating that they do not have a critical role in motility. On the other hand, although strain 680 (ST8) has a group of *fli* genes in its genome, this however did not show motility in the laboratory experiment (Fig.3.1, Table.3.4). Moreover, it was the only strain that showed the absence of *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliK*, *fliL*, and *fliM* genes (Table.3.1). This confirms the predicted annotation based on whole genome sequencing that these genes might be those essential for the expression and the movement of the flagella as their absence affected the ability of the strain to be motile (Fig.3.1, Table.3.4). However, the lack of functional genomic analysis studies regarding this organism keeps the functions of several genes unresolved and further testing is needed to clarify their roles.

Motility is important for the bacteria to aid the invasion of the tissues in order to overcome the host barriers. Moreover, it contributes to the translocation of strains through the damaged tissues to infect the healthy ones, which causes more damage to the host. The non-motile ST4 strains 1223 and 1224 showed moderate levels of attachment, invasion, and translocation through Caco-2 and HBMEC cell lines. However, strain 1223 showed higher translocation level through the HBMEC cell line indicating that this strain is capable of infecting this cell line even with the lack of

ability to move, and the strain might depend on non-flagellar mediated motility using different bacterial traits such as fimbriae (twitching motility), which indicates the virulence potential of this strain. Strains 1223 and 1224 were clinical isolates from blood, however they were not linked to fatal cases and they did not translocate through BBB to the brain *in vivo*, as there were no meningitis cases reported (Table.3.4). On the other hand, strain 680 is a CSF isolate though not associated with fatal infection, and was expected to have a high virulence potential based on the site of isolation. However, this strain showed low attachment, invasion, and translocation through Caco-2 and HBMEC cell lines. Furthermore, it demonstrated low attachment and invasion for rBCEC4 cell line. Strain 680 (ST8) was rapidly killed by macrophages and microglia. The low attachment and invasion profiles of this strain might be due to the lack of flagellar motility that aids adherence and invasion (Table.3.4-5). Thus, the presence of functional flagella that allows the organism to move is one of the important traits that contributes to *C. sakazakii* virulence.

3.4.2. Iron siderophore detection

Iron is an important microelement and it is essential for many bacterial cellular processes. These include cellular respiration and superoxide metabolism in addition to its role in pathogenesis (Tanaka *et al.* 1994, Yoshida *et al.* 1995, Bishop *et al.* 2011). As a part of the immune response, the human body reduces the iron availability to decrease the free iron levels that will limit bacterial growth (Parrow *et al.* 2013). Moreover, as a part of acute-phase response (APR), which is initiated by the innate immunity, a striking change in iron metabolism occurs in response to infection. The aim of APR is to eliminate the source of infection and minimise the damage (Epstein *et al.* 1999). Moreover, during APR the intracellular storage of iron in ferritin is increased as in some cells including microglia and macrophages. Additionally, under aerobic conditions, iron is present as insoluble and biologically inaccessible ferric hydroxide (Fe^{3+}). In such iron-limited environments, bacteria produce high-affinity iron binding molecules, such as siderophores, to acquire iron from these environments. By forming water-soluble hexadentate ferric complexes, siderophores can bind to the 6 coordinate

sites of ferric ions, as siderophores are usually classified by the ligands used to chelate the ferric iron. There are 3 major groups of siderophores including catecholates-phenolates such as enterobactin, hydroxamates such as aerobactin, and carboxylates such as citric acid (Neilands 1981, Yoshida *et al.* 1995, Miethke and Marahiel 2007, Franco *et al.* 2011a, Grim *et al.* 2012, Parrow *et al.* 2013). The presence of different iron acquisition systems and iron transporters is important for pathogens, including *C. sakazakii*, to enable efficient iron acquisition to survive in iron-limited environments in various niches, which could support bacterial pathogenesis (Grim *et al.* 2012). Moreover, this might help the bacteria to uptake the iron from the iron-rich cells in human brain such as microglia (Barron 1995).

In this research, *C. sakazakii* strains were tested using CASAD for their ability to produce iron siderophores that could help in iron acquisition, which in turn is a virulence factor. All the strains showed an ability to produce these molecules, except for the plasmid-less strains 6 (ST4) and 520 (ST12; Fig.3.2). The ability to produce such molecules gives the advantage to the bacteria to survive in the human body and support pathogenesis, as iron is an important biological cofactor.

The *C. sakazakii* genome encodes for iron acquisition genes including *eitA*, *iucC*, and *viuB*. The gene *eitA* is a part of *eitCBAD* operon that shows homology with ABC transporters involved in iron, siderphore, and haem uptake, while *iucC* that is a part of *iucABCD* iron uptake system, which is responsible for cronobactin biosynthesis and transport that mediates *Cronobacter* spp. growth under iron limiting conditions. The gene *viuB* has a role in the aerobactin system as a siderophore exporter (Franco *et al.* 2011a, Grim *et al.* 2012). Although the majority of strains harboured these genes, the plasmid-less strains 6 (ST4) and 520 (ST12) were negative for them. The plasmid PCR profile showed that these strains lack these genes because they do not have the large pESA3 *C. sakazakii* plasmid. Moreover, the genomic comparison confirmed the absence of these genes in their genomes and their presence in the other strains (Table.3.2). The analysed strains harbouring *eitA*, *iucC*, and *viuB* have the

ability to acquire iron from iron limited environment by producing iron siderophores, which help in the survival in the host and overcome the immune response that aims to limit the iron levels at the time of infection (Franco *et al.* 2011a, Grim *et al.* 2012). The production of siderophore was confirmed using CASAD (Fig.3.2). On the other hand, strain 557 (ST4) was negative for *eitA* and positive for *iucC*, and strain 12 (ST1) was positive for *eitA* and negative for *iucC*. In addition, strain 696 lacked the presence of *viuB* gene. This, however, did not affect their ability to produce iron siderophores (Table3.2 and Fig.3.2). This might be attributed to the presence other siderophore-related genes that were not examined in this project

3.4.3. *C. sakazakii* sensitivity to human serum

Invasive microorganisms have protective mechanisms against serum-mediated killing. Bacterial structures including outer membrane proteins and proteases were identified for their roles to avoid this bactericidal action (Taylor 1983, Rautemaa and Meri 1999, Schwizer *et al.* 2013). A recent study by Franco *et al.* (2011b) showed that the *Cronobacter* outer membrane protease (Cpa) is a plasminogen activator that plays an essential role in serum resistance. Moreover, it was reported that the mucoid appearance of *E. coli* K12 at 37°C was attributed to the presence of the *rcsA* gene product (McCallum and Whitfield 1991). It has been shown recently that colanic acid provides protection against the bactericidal effect of human serum in *E. coli* (Li *et al.* 2005, Miajlovic *et al.* 2014).

A group of *C. sakazakii* strains in this research (*n*=13) were tested for their ability to resist human serum. About 85% of strains were regarded as resistant and able to replicate in human serum and they appear to be completely refractory to serum killing as were *Cit. koseri* and *S.* Enteritidis the positive controls. *C. sakazakii* strains 6 (ST4), 680 (ST8) and *E. coli* K12 the negative control were sensitive and underwent significant reduction in viability (*P*<0.05; Fig.3.3). Withstanding serum killing is an important factor that leads to the survival in the host blood and might participate in causing bacteraemia.

A previous study by Franco *et al.* (2011b) showed that the *cpa* mutant of strain 658 (ST1; BAA-894) was serum sensitive compared with the wild type. In this project, *C. sakazakii* clinical strains, including meningitis isolates, were examined for their ability to survive in human serum in the presence and absence of *cpa* and *rcsA* genes that might confer serum resistance in *C. sakazakii*. Eighty five percent of the strains that were tested for serum sensitivity and encode the *cpa* gene (*n*=11) were able to resist serum bactericidal activity, whereas the strains that were negative for *cpa* (*n*=2) showed serum sensitivity (Fig.3.3 and Table.3.2). Another gene, *rcsA*, responsible for colanic acid production was detected in all sequenced strains except for strains 680 (ST8) and 520 (ST12; Table.3.2). The expression of colanic acid confers serum resistance in *E. coli* as reported previously (McCallum and Whitfield 1991, Li *et al.* 2005). Strain 680 (ST8) that is serum sensitive lacks *cpa* and *rcsA* genes that are possible genes responsible for serum resistance (Fig.3.3 and Table.3.2). On the other hand, strain 6 (ST4) is another serum sensitive strain that encodes the *rcsA* gene but lacks the *cpa* gene. Despite the presence of the *rcsA* gene that might be responsible for serum resistance, it showed serum sensitivity. Moreover, Bogard and Oliver (2007), reported that the *viuB* gene has an important role in serum resistance in *Vibrio vulnificus*, as the resistance is influenced by serum iron availability. They found that the strains positive for *viuB* showed significant serum resistance, while strains lacking this gene were sensitive. In the case of *C. sakazakii,* the presence of *viuB* by itself does not affect serum resistance, as strain 680 (ST8) harboured *viuB* and lacked *cpa* and showed serum sensitivity (Fig.3.3)*.* On the other hand, the strains that have both genes such as strains 767 (ST4) and 658 (ST1), and strain 696 (ST12) that has *cpa* and lacked *viuB* displayed resistance to human serum, suggesting that *cpa* gene has the leading role in the resistance (Fig.3.3).

The previous results suggest that *cpa* is an important key factor in *C. sakazakii* serum resistance. Although the data showed that the serum resistance was linked to the presence of *cpa* gene, they do not nevertheless indicate that the resistance is a property exclusively

dependent on this gene. There might be other factors that contribute to the resistance.

3.4.4. Bacterial cytotoxic activity to human cell lines (MTT)

Cytotoxicity is an indicator of the virulence of the organism. This can help the organism to overcome the physical barriers of the host by causing cell death, hence altering the tight junctions and making more passages for more bacterial cells to invade. MTT assay is one of the tests that can measure the cytotoxic effect on eukaryotic cells. It shows the ability of the mitochondria of the healthy cells to reduce MTT to its insoluble purple form formazan (Fotakis and Timbrell 2006, Sharma *et al.* 2009, Travan *et al.* 2009). In this experiment a washing step with PBS was added before adding the MTT to remove the unattached extracellular bacterial cells to minimise the possibility of false positive results.

A group of *C. sakazakii* strains in this project (*n*=13) were tested for their cytotoxic impact using MTT assay. All those strains were able to induce cell death of the Caco-2 cell line, as the assay displayed declined absorbance levels after 3 hours of incubation indicating low MTT conversion (the lower absorbance the higher cytotoxicity). CC4 strains 20, 695, 1242, 1587 in addition to 658 (ST1) and *S.* Enteritidis were the most cytotoxic strains tested (*P*<0.05; Fig.3.5). This suggests that CC4 strains (average absorbance after 3 hours of infection 0.177) were able to induce cytotoxicity more than the non-CC4 strains (average absorbance after 3 hours of infection 0.198) used in this experiment, indicating their virulence potential for this cell line. Moreover, all these strains showed the ability to translocate through the Caco-2 cell line and translocation was accompanied by severe drops in the transepithelial electrical resistance due to disruption of the tight junctions (Fig3.17-19 and Table.3.5). It is proposed that the ability of these strains to cause cytotoxicity of the Caco-2 cell line might alter the tight junctions and facilitate their translocation through the intact monolayer. This process mimics the mechanism that the organism might use to invade the intestinal epithelial barrier *in vivo.* This could lead to a loss in the integrity of the intestinal mucosa leading to the permeability of this barrier allowing the bacterium to migrate through

this disrupted layer to the tissues beneath. This could then cause more damage to the healthy tissues of the gut and allow the organism to reach the blood vessels. One *C. sakazakii* strain known to cause a fatal NEC II case in the French outbreak in 1994 (Caubilla-Barron *et al.* 2007), which is 695 (ST4), was able to induce cell death and translocate through Caco-2 cell line suggesting its ability to overcome intestinal barriers *in vitro* and *in vivo*. On the other hand, strain 6 (ST4) showed low cytotoxicity and was not able to translocate through Caco-2 cell line. In addition, strain 558 (ST4) exhibited low cytotoxicity and translocation suggesting that the ability of the organism to cause cytotoxicity is important to allow invasion of the Caco-2 cell line (Table.3.3).

With regard to the HBMEC cell line, it did not show susceptibility to cytotoxicity over the first hour of the assay in contrast to Caco-2 cells; nevertheless after prolonged incubation for 3 hours the cytotoxic effect appeared to be increased. CC4 strains 20, 695, 767, 1221, 1240, and 1587 in addition to strains 658 (ST1), 696 (ST12) and *Cit. koseri* demonstrated high cytotoxicity when compared with the negative control ($P<0.05$; Fig.3.6). These strains except strain 1587 were only able to pass through the polarised monolayer of HBMEC cell line 5 hours after infection, indicating that this cell line is less susceptible to translocation than Caco-2. Additionally, the transendothelial resistance displayed a decline after 5 hours of incubation (Fig.20-22 and Table.3.5). Despite the fact that the decrease in TEER levels was not significant, it indicates that there was a disruption in the tight junction that might be attributed to the cytotoxicity of these strains for the HBMEC cell line. Strain 767 (ST4) that was linked to a fatal neonatal meningitis case (Caubilla-Barron *et al.* 2007) showed high cytotoxicity and translocation ability through the HBMEC cell line. On the other hand, ST4 strains 6 and 558 were low in cytotoxicity and translocation indicating their low virulence potential (Table.3.5).

Although the strains were ultimately cytotoxic for the cell line, the mechanism of initial HBMEC resistance to cytotoxicity remains unknown. Hence, it is important to understand the factors beyond this mechanism.

Ramegowda *et al.* (1999) showed that HBMEC treatment with TNF-α and IL-1β resulted in increased expression of the toxin-binding glycolipid globotriaosylceramide and made the cells sensitive for *Shigella* cytotoxicity. Another study by Eisenhauer *et al.* (2001) reported that HBMEC cells become more sensitive to Shiga toxin and susceptible to the damage in response to elevated TNF-α levels. *Shigella* and *C. sakazakii* belong to the *Enterobacteriaceae* family, and *C. sakazakii* strains might induce similar responses in HBMEC cells by the stimulation of TNF-α production, which in turn makes the cell line more susceptible to *C. sakazakii* cytotoxicity. Cruz-Córdova *et al.* (2012) reported that *C. sakazakii* flagella were able to induce TNF-α production. Moreover, HBMEC cell line was induced to produce TNF-α by *C. sakazakii*, and the induction of this cytokine was detected over 5 hours of infection (data will be shown later in Section 4.3.3.1 Fig.4.5). This increase in TNF-α might potentiate the susceptibility of these cells to cytotoxic killing by *C. sakazakii* as shown above (Fig.3.6).

Regarding the HMGC cell line, most of *C. sakazakii* strains were able to cause a significant cytotoxic effect and cell death ($P<0.01$) including CC4 strains 20, 695, 767, 1221, 1240, and 1587 in addition to strains 1249 (ST31), 696 (ST12) and *Cit. koseri*. These strains showed the ability to multiply and survive within microglia especially the CC4 strains and 1249 (ST31), which showed the highest survival and multiplication (Fig.3.25). The CC4 strains (average absorbance after 3 hours of infection 0.15) showed higher cytotoxicity for HMGC than the non-CC4 strains (average absorbance after 3 hours of infection 0.172). It is important to understand the mechanism whereby *C. sakazakii* could cause this massive cytotoxic effect to HMGC. This is currently unknown due to the lack of publications showing the bacterial effect on this unique type of immune cells. *C. sakazakii* might also be able to cause such an effect as a result of the survival within these cells and production of toxins intracellularly, and therefore would not need any toxin receptors on the cell surface.

It was shown by Kim *et al.* (1995) that pneumococci are toxic to microglia and this toxicity is likely to be mediated by the pneumococcal cell wall.

Although, *C. sakazakii* is a Gram-negative bacterium in contrast to *S. pneumoniae*, which is Gram-positive, it is speculated that *C. sakazakii* LPS, as an outer membrane component, might play a role in inducing cytotoxicity and cell death via apoptosis. It was proposed by Liu *et al.* (2001) that over activation of rat microglia by a high concentration of LPS resulted in apoptotic death of rat microglia shown by DNA fragmentation, phosphatidylserine expression, and caspase-3 activation. In addition, they showed using the MTT assay that LPS-induced cytotoxicity to microglia was concentration- and time-dependent. They demonstrated that the exposure of rat microglia to a high concentration of LPS led to decreased microglial viability after 1 hour, whereas a low concentration of LPS such as 1 ng/ml did not affect the viability over 24 hours (Liu *et al.* 2001) Moreover, the cytokines released at the time of the infection might contribute to inducing cell death. All these mechanisms need to be examined and clarified in order to understand the behaviour of the organism in this phenomenon, as the ability to induce cytotoxic killing in this kind of cells is a major threat to the human CNS.

In contrast, strains 6 (ST4), 558 (ST4), 680 (ST8), and *E. coli* K12 were the least cytotoxic among all tested cell lines. These strains showed very low invasive and translocation abilities, and low phagocytosis survival when incubated with human cell lines indicating their low cytotoxicity (Table.3.4 and Table.3.5).

3.4.5. *C. sakazakii* attachment, invasion, and translocation assays

Tissue culture assays were applied to examine the bacterial-host interaction and to assess the ability of *C. sakazakii* strains to overcome human intestinal and brain barriers represented by Caco-2 and HBMEC cell lines. Moreover, it tested the ability of these strains to attach and invade rat brain microvascular endothelial cells represented by the rBCEC4 cell line. These experiments included attachment and invasion (gentamicin protection) assays in addition to translocation assay.

C. sakazakii showed the ability to attach to the three cell lines that were used in this research indicating its virulence potential. Some strains such as 696 (ST12) and CC4 strains 695, 767, and 1465 exhibited higher levels

of attachment to Caco-2 cells ($P<0.05$), whereas strain 696 (ST12) in addition to CC4 strains 695, 767, and 1221 showed elevated attachment levels to HBMEC cells ($P<0.05$). Furthermore, *C. sakazakii* strains showed the ability to attach to rBCEC4 cell line and CC4 strains 20, 695, 767, 1221, and 1587 in addition to 1249 (ST31) and 696 (ST12) showed highest attachment among strains ($P<0.01$). The attachment ability of the organism to this line was examined to demonstrate whether the bacterium will behave similarly with rat and human cells. The ability of *C. sakazakii* strains to attach to rat brain cells was slightly higher than for human cells; this however does not indicate that the invasion ability will be higher. The number of bacterial cells of CC4 strains (average attachment efficiency for HBMEC 2.4% and rBCEC4 2.9%) attached to both cell lines was higher than those of non-CC4 strains (average attachment efficiency for HBMEC 1.5% and rBCEC4 2.6%) indicating their potential to invade these cell lines. A previous study by Townsend *et al.* (2008) applied the attachment assay on a group of strains including 695 (ST4), 767 (ST4), and 696 (ST12) using the Caco-2 cell line. Their results confirmed the ability of the strains to attach to the cell line, but the attachment levels for these strains were 25-75% lower when compared with the results obtained in this research. Although these results showed the ability of these strains to attach to Caco-2 and HBMEC cell line, this however does not necessarily indicate their invasion ability.

The attachment process is the first step of the invasion process, thus the invasion assay was conducted to investigate their ability to invade the previously mentioned cell lines. All the strains were able to invade Caco-2 cells albeit that some strains displayed different invasion levels. For example, strain 695 (ST4), which showed high cytotoxicity for Caco-2 cells (Table.3.5 and Fig.3.5), showed highest invasion ($P<0.05$), whereas strains 6 (ST4), 4 (ST15), 12 (ST1), 1 (ST8), and 5 (ST8) showed the lowest invasion levels. The other strains showed moderate invasion capacity (Table.3.4 and Fig.3.13). The invasion results were compared to the results obtained by Townsend *et al.* (2008), and the invasion levels for the strains 695 (ST4; $P<0.05$), 767 (ST4), and 696 (ST12) were higher by 15-85% than the levels published in that previous study. The differences

in the results of the attachment and invasion experiments obtained from this research and the previously published study by Townsend *et al.* (2008) might be because of the differences in strains growth conditions, as the study grew the strains tested on TSA plates and then harvested them directly from the plate in tissue culture medium. Moreover conditions such as cell line confluency, the passage number of the cell line, the incubation conditions, and the concentrations of the reagents used such as the concentration of Triton X-100 solution might contribute to obtaining different results, but both confirmed the ability of the strains to attach and invade the cell line.

With reference to HBMEC cell line invasion, most of the strains showed moderate invasion and strain 767 (CC4) displayed a significantly higher invasion level (*P*<0.01). Nevertheless, strain 6 (ST4) was not able to invade while the other strains such as CC4 strains 553, 557, 558, 1465, and 4, in addition to 12 (ST1), 555 (ST1), 658 (ST1), 680 (ST12), and 1 (ST8) showed low invasion levels (Table.3.4 and Fig.3.14). Regarding rBCEC4 cell line invasion, strains 1221 (CC4), 1587 (CC4), and 696 (ST12) showed highest invasion (*P*<0.05), whereas strains 6 (CC4), 558 (CC4), 658 (ST1), and 680 (ST8) showed the lowest levels (Table.3.5 and Fig.3.15). It was notable that the invasion of HBMEC is 10 times higher than the invasion of rBECE4 cells suggesting that the organism has the tendency to invade human cells more that the rat ones. Townsend *et al.* (2007b) examined the ability of *Cronobacter* strains to invade the rBCEC4 cell line. This corresponds with our results, however strain 658 was more invasive in the previous published work. CC4 strains (average invasion efficiency for HBMEC 0.13% and rBCEC4 0.02%) were able to invade HBMEC and rBCEC4 cell lines at high levels in comparison to non-CC4 strains (average invasion efficiency for HBMEC 0.1% and rBCEC4 0.016%). Nevertheless, the ability of strains to invade human cells was much higher than rat cells (Table.3.5).

A group of invasion-related genes were examined for their presence in *C. sakazakii* genomes of the sequenced strains analysed in this project. These genes include *apaH*, *ompA*, *ompX*, *ygdP*, and *ppk1*. ApaH is a

dinucleoside polyphosphate hydrolase that cleaves Ap$_4$A producing 2-adenosine diphosphate (2ADP). It was reported that *apaH* has a role in the invasion of human epithelial cells by *S*. Typhimurium, and *apaH* mutants showed 2-fold reduction in their invasion (Ismail *et al.* 2003). Additionally, it was shown that the deletion of *apaH* resulted in reduced motility in *E. coli* (Farr et al. 1989, Ismail et al. 2003). It was reported that the outer membrane protein A (OmpA) of *Cronobacter* spp. has 88% similarity to that of *E. coli* K1 at the protein level. This protein promotes the invasion ability of *E. coli* K1 to human intestinal and brain mircovascular endothelial cells *in vitro* (Singamsetty *et al.* 2008, Mohan Nair *et al.* 2009). It was also shown that the expression of OmpA is critical for *Cronobacter* spp. invasion of HBMEC and is required for microtubule condensation, PI3-kinase, and PKC-α activation (Singamsetty *et al.* 2008). Kim *et al.* (2010) demonstrated that OmpA and OmpX are essential for *Cronobacter* basolateral invasion of host cell including Caco-2 and INT407 cells, and the movement into deeper organs. Additionally, the invasion levels of *ompA* and *ompX Cronobacter* mutants to Caco-2 cell line were decreased compared to the wild type. However, the invasion of *ompX* mutant was higher than *ompA* mutant suggesting that OmpA has a dominant role in Caco-2 invasion.

It has been demonstrated by Badger *et al.* (2000) that the *ygdP* gene might be involved in HBMEC invasion by *E. coli* K1. Their results showed that *ygdP* mutant showed decreased HBMEC invasion compared with the wild type. YgdP has been identified as a member of the superfamily of Nudix hydrolases that catalyses the hydrolysis of the diadenosine polyphosphates Ap$_4$A, Ap$_5$A, and Ap$_6$A. These molecules are involved in cellular responses, and the concentration of these molecules can increase 100-fold over their regular levels following heat shock or oxidative stress. It was hypothesised that YgdP might function by decreasing the concentration of these signaling molecules during the invasion process (Bessman *et al.* 2001).

Polyphosphate kinase 1 (PPK1) that is encoded by the *ppk1* gene is an enzyme responsible for the synthesis of the inorganic polyphosphate from

adenosine triphosphate (ATP). In addition, it was found to be important in stress adaptation and the expression of the virulence genes of *E. coli* K1 including *ompA*. It was shown that the deletion in *ppk1* in the mutant led to a decrease in the adhesion and invasion of HBMEC, meningitis development, and TNF-α and IL-1β production in a newborn rat model (Peng *et al.* 2012).

All *C. sakazakii* sequenced strains in this research (*n*=24) were positive for *apaH*, *ompA*, *ompX*, *ygdP*, and *ppk1* (Table.3.2). Despite the presence of these genes the strains vary in their invasion ability (Fig3.13 and Fig.3.14). ST4 strains 6, 721 in addition to strains 1 (ST8), and 5 (ST8) showed low invasion levels to Caco-2 cell line (Fig.3.13). Furthermore, ST4 strains 553, 557, 558, and 730 in addition to strains 4 (ST15), 658 (ST1), 1 (ST8), 5 (ST8), 680 (ST8), and 520 (ST12) were low in invasion to HBMEC cell line (Fig.3.14).

The presence/absence of these genes does not correlate with the variable invasion profiles indicating that these genes are not essential for *C. sakazakii* invasion to HBMEC. The invasion process is multifactorial and involves different bacterial traits such as flagella. This can be seen in the case of strains 695 and 767 (CC4; Fig.3.13-14 and Table.3.5) that were positive for all invasion genes and demonstrated high invasion capacity in addition to being associated with fatal NEC and fatal neonatal meningitis respectively (Caubilla-Barron *et al.* 2007). On the other hand, other strains such as 6 (CC4) and 558 (CC4) that are positive for invasion genes showed low invasion levels (Table.3.5).

As highlighted before, some CC4 strains did not follow the same behaviour of attachment and invasion of the clonal complex. Strain 1465 (CC4) that was isolated from powdered infant formula showed high attachment to Caco-2 and HBMEC cell lines and low invasion, indicating that the ability to attach might not necessarily lead to high invasion. Moreover, strains 6, 553, 557, 558, and 4 exhibited low attachment, invasion, and translocation levels. Although these strains are clinical isolates, their association with any clinical presentations and isolation sources are unknown. They might not be associated with severe infections or may be

from faecal samples indicating their colonisation of the gut. Additionally, they might harbour non-functional virulence genes that require further examination using functional genomics' tools. Furthermore, it was noted that some clinical isolates behaved differently when examined *in vitro* and showed low virulence compared with the deleterious clinical outcomes of the patients. For example, strain 1019 (ST1) and the non-motile *C. sakazakii* strain 680 (ST8), which are CSF isolates, demonstrated low invasion and translocation ability for Caco-2 and HBMEC cell lines. In addition strain 680 (ST8) was sensitive to human serum and showed low phagocytosis survival. Furthermore, during the period of this project a *C. malonaticus* clinical isolates was received from the CDC. This strain (1569 ST112) was isolated in 2011 from the blood of <1-month-old infant who died as a result of fatal meningitis infection (Joseph and Forsythe 2012c). It is the first neonatal meningitis case that has been attributed to this species. The low attachment, invasion, and translocation results *in vitro* of this strain did not correlate with the severe outcome of the infection (for results refer to Appendix.1). This suggests that some strains could behave differently inside the host and this might be attributed to some host factors that could be unavailable *in vitro*.

Overall, the invasion results indicated that the majority of the strains were able to invade the cell lines and therefore potentially translocate towards the underlying tissues and blood stream, especially CC4 strains, leading to the dissemination of the organism all around the body. Hence, translocation assays using the same human cell lines were applied to investigate the ability of the organism to translocate through these cell lines. Most of the strains, including those of CC4, were able to translocate through Caco-2 cells especially strains 695 (CC4), 658 (ST1), and 696 (ST12) that displayed significantly higher translocation ability ($P<0.01$; Fig.3.17). These strains were also highly cytotoxic for Caco-2 cells, which could lead to an increase in the cell line permeability causing more bacterial translocation as mentioned previously. Nonetheless, strains 6 (CC4), 1019 (ST1), 1 (ST8), and 5 (ST8) were not able to translocate, and strain 6 (CC4) showed low cytotoxicity (Table.3.5 and Fig.3.5). Thus, the translocating isolates potentially have the advantage to overcome the

gut barriers and cause more damage to the host, whereas the non-translocating strains lack this advantage. Strain 695 (ST4) accounted for a fatal neonatal NEC infection while strain 696 (ST12) was previously linked to neonatal NECII infection (Caubilla-Barron *et al.* 2007). The invasion and translocation results correlate with the virulence potential of these strains and their ability to cause that type of disease.

The transepithelial electrical resistance declined when using the invasive strains, especially the strains that were able to cause high cytotoxicity; strains 695 (CC4), 658 (ST1), and 696 (ST12). This explains the mechanism of translocation for the strains where they can migrate in between the cells. TEER, however, remained steady when using the non-translocated isolates. However, a group of strains were able to translocate through Caco-2 monolayers without any notable change in TEER. These strains include CC4 strains 20, 553, 557, 558 730, 1219, 1221, 1231, 1240, 1242, 1465 in addition to 680 (ST8), and 580 (ST18). They might use different translocation mechanism other than the disruption of the tight junctions. The translocation might be transcellular, whereby the bacterial cells invade and pass through mammalian cells to basolateral compartment, as suggested previously by Giri *et al.* (2011). Thus, it is proposed that *C. sakazakii* could translocate through Caco-2 monolayers using two different mechanisms; paracellular (between cells) and transcellular (through cells). Most of the CC4 and non-CC4 strains were able to accomplish the translocation process through Caco-2 monolayers indicating their ability to overcome the physical barriers of the human gut, and there was no advantage for CC4 strains over the non-CC4 ones.

The above experiments suggested that some bacterial strains were able to disrupt the tight junctions of Caco-2 cells that helped in their translocation. Most of them were able to induce cytotoxic killing, which illustrates the impact of cytotoxicity on the cell line (Table.3.5 and Fig. 3.5). The cytotoxicity and translocation might trigger the onset of NEC, and could lead to the spread of the bacteria in the blood stream. This dissemination of the organism suggested that it would be able to reach the brain microvascular endothelium and invade its cells. The results of

the invasion assay support the proposed theory, and therefore the translocation assay using HBMEC cell line was initiated to investigate whether the organism has the ability to translocate through these cells or not. CC4 strains 20, 695, 767, 1219, 1220, 1221, 1222, 1231, and 1240 in addition to strain 696 (ST12) exhibited the highest translocation levels ($P<0.01$), while CC4 strains 6, 553, 558, and 1465, and 4 in addition to 12 (ST1), 1 (ST8), and 5 (ST8) did not translocate. The other strains showed low to moderate translocation ability.

Strains 695 (CC4), 767 (CC4), and 696 (ST12), which were the highly translocated strains showed high cytotoxicity to HBMEC cells (Table.3.5 and Fig. 3.6), and were linked to severe and fatal infant infections in the French outbreak in 1994 (Caubilla-Barron *et al.* 2007). Strain 767 (CC4) was associated with a fatal meningitis case and strain 695 (CC4) was responsible for fatal NEC II infection. However, strain 696 (ST12) was linked to a non-fatal NEC II infection (Caubilla-Barron *et al.* 2007). Strains such as 1465 (CC4) from infant formula, 12 (ST1) from faeces sample, and 1 (ST8) from throat sample are non-translocating isolates and were not linked to any fatal neonatal infections (Table.3.4). It was clear that the most of the translocating strains belong to CC4, which is the lineage that is linked to the most neonatal meningitis cases worldwide (Joseph and Forsythe 2011). These stains (average translocation efficiency for HBMEC 4.92%) were able to translocate through HBMEC cells more than the non-CC4 strains (average translocation efficiency for HBMEC 1.67%). ST4 strains such as 767, 1221, 1240, and 1242 among CC4 strains were invasive and highly translocated isolates and were responsible for fatal meningitis infections. This might indicate their potential to translocate through BBB *in vivo* and cause meningitis. It is important to study these strains and discover their role in the triggering the host response and its outcome.

The HBMEC transendothelial electrical resistance (TEER) was largely stable during the first 4 hours of incubation, indicating the stability of the tight junctions and the integrity of the cell line (Fig.3.21). However, after 5 hours of infection TEER showed declined levels with some strains that

were able to translocate such as strain 767 (CC4), which translocated to the highest level among *C. sakazakii* strains ($P<0.001$; Fig3.20). The drops in TEER are attributed to the deterioration of endothelial integrity that could be accredited to the cytotoxic killing of the HBMEC cell line that was caused by those strains. According to the TEER results it is proposed that HBMEC cell line is less susceptible to cytotoxicity than the Caco-2 cell line, as the latter showed considerable decrease in resistance values (Fig.3.18).

The translocated strains might need a longer infection period to accomplish the translocation process, as the translocation was noted 5 hours post infection, while in Caco-2 it was after 1 hour with some strains. Moreover, they could use different mechanisms of invasion to overcome this barrier that is a part of the BBB. One of the possible mechanisms that might assist the strains to translocate is the bacterial cytotoxicity that initiates cell death in the cell line via apoptosis or necrosis. Moreover, the cytokines released by the cells may play a role in the same process by making the cell line permeable. High levels of NO are a potential factor that might contribute to the permeability of the cell line. The translocation of these strains indicated their potential to pass through towards the brain tissues triggering the host response, which could result in brain inflammation and tissue damage.

In a previously published study by Giri *et al.* (2011), non-clinical *C. sakazakii* isolates were examined and showed their ability to invade and translocate through Caco-2 and HBMEC cell lines. However, the translocation results obtained in our research demonstrated higher ability of *C. sakazakii* strains to invade and translocate through the same cell lines. Most of the strains that were used in our research were from clinical sources and were linked to severe and fatal cases (Table.3.4). This indicates the virulence potential of the clinical strains over the non-clinical ones and this will enable us to apply more assays to investigate the impact of our clinical strains on the host and to elucidate the host cell response to infection.

Choosing human cell lines is important to focus on the impact of *C. sakazakii* infection on the human host and its outcome, as the bacterium showed a greater capacity to invade those cells over the rat ones. This could help to clarify the pathogenesis of the diseases that are caused by this organism and might aid the treatment. Moreover knowing the history of *C. sakazakii* strains could help in understanding their virulence potential. The strains that showed moderate to high invasive ability were isolated from clinical sources and were linked to severe cases (Table.3.4-5). Most of these strains belong to CC4, which includes STs such as ST4, ST15 and ST109. For example, strain 695 (ST4) and 767 (ST4) accounted for fatal NEC II and neonatal meningitis infections respectively. On the other hand, strain 1465 (ST4), which was isolated from PIF, displayed low invasion and was not linked to any of the serious clinical presentations such as meningitis or NEC. Moreover, there were some clinical strains such as ST4 strains 6 and 558 that were isolated from clinical sources and showed low invasion and translocation ability. However, the clinical presentation of these strains was unknown. One of the reasons behind their decreased virulence might be accredited to losing some virulence genes as in the case of strain 6, which showed the absence of large *C. sakazakii* plasmid pESA3 that harbours a group of important virulence genes such as *cpa* that confer serum resistance (Table.3.2). The history of these strains could provide a solid base for expanding the research and include new experiments that in turn will provide a clearer picture about the relationship of the isolates and the host response in terms of infection.

3.4.6. Uptake and survival assays inside phagocytic cells

The survival studies were conducted to investigate the ability of the test strains to survive and multiply within macrophages and microglia that are types of immune cells. *C. sakazakii* strains used in the survival experiments (*n*=13) showed the ability to persist within human macrophages cell line U937 cell line for up to 72 hours of incubation. Moreover, CC4 strains 20, 695, 767, and 1221 in addition to 658 (ST1) were able to survive and multiply significantly compared to other *C. sakazakii* strains (*P*<0.05). The survival results were compared to the results obtained previously by Townsend *et al.* (2008). The results

obtained in this project partly confirmed the results that were reported in the previous publication. Strains 767 (CC4) and 696 (ST12) showed similar persistence and multiplication levels as the *C. sakazakii* strains used in that research. However, Townsend *et al.* (2008) reported that strain 695 (CC4) was able to survive but could not multiply within macrophages. These results are in contrast to those obtained in this project as strain 695 (CC4) showed the ability to survive and multiply in U937 cells. Our results correlate the virulence potential of the strain with the attachment, invasion, and translocation profiles, as it was an invasive strain for both Caco-2 and HBMEC cell lines. Moreover, it is a clinical strain that was responsible for a fatal NEC infection (Table.3.4). This suggests that this strain was able to establish a successful infection and has the required tools for this process including avoiding phagocytic killing.

The survival and multiplication within macrophages could help the organism to use macrophages as a vehicle to invade the other body organs. This mechanism that is called "Trojan horse" is where the organism translocates through tissues inside macrophages (Fig.2.3). This mechanism allows the bacterium to hide inside the phagocytic cells, escape from the immune response, and reach the other body organs such as the brain. Some cytokines secreted by the infected tissues attract phagocytic cells and make these tissues permeable and leaky to allow this kind of the immune cells to migrate to the site of infection, and help in increasing the number of the invading organism. The damage can be indicated by the host response induced by the bacterium. Moreover, the persistent strains were confirmed to be serum resistant (Table.3.4 and Fig.3.4), and these two characteristics could enhance their ability to avoid the host immune response and cause bacteraemia, which could be advantageous for the organism to migrate through the BBB endothelium.

Microglia are brain resident innate immune cells that are responsible for phagocytosis besides their ability to produce inflammatory mediators such as NO and TNF-α. The ability of these cells to eliminate *C. sakazakii* CNS infection *in vitro* has not yet been examined. It was shown in this research that the majority of *C. sakazakii* test strains were able to persist in human

microglia, as represented by the HMGC cell line, for 72 hours. Moreover, CC4 strains 20, 767, 1221, 1240, and 1587 in addition to 1249 (ST31), which were highly toxic to HMGC (Table.3.5, Fig.3.7, and Fig.3.26), multiplied significantly in this cell line ($P<0.01$). Although these strains showed the ability to multiply within microglia, they nevertheless showed declined levels of persistence afterwards. This might be related to the high cytotoxicity that led to the death of microglia causing low viable count at the last two time points of the assay (48 and 72 hours; Fig.3.25). On the other hand, the strains that were not able to multiply and were killed rapidly such as strains 6 (ST4), 558 (ST4), and 680 (ST8) were low in cytotoxicity (Table.3.5). A correlation was found between the cytotoxicity of the organism and the ability to survive and multiply. It was found that the strains that showed high cytotoxicity to the HMGC cell line such as CC4 strains 767 and 1587 were high in persistence and multiplication within microglia (Table 3.5, Fig.3.7, and Fig.3.25). Sixty seven percent of the replicating strains belong to CC4, which is the lineage responsible for most of the CNS infections among *C. sakazakii* isolates (Joseph and Forsythe 2011). It was notable that the multiplication of the strains, which showed high cytotoxicity, was followed by a remarkable drop in their viable counts. Thus, it is proposed that this drop could be attributed to the cell death due to high cytotoxicity that might have led to the loss of the bacterial cells that were in those cells after the washing step. The ability to reproduce intracellularly in these phagocytic cells by this pathogen indicates their virulence potential allowing them to withstand the bactericidal activity of microglia and evade the host immune response.

Research by Liu and Kielian (2009) showed the ability of *Cit. koseri* to survive and multiply within microglia and U937 cell lines. Therefore, this organism that is responsible for fatal CNS infections was used as a positive control for this experiment. It showed high ability to survive and multiply in HMGC cells, which confirmed the survival potential reported in the previous research. On the other hand, *C. sakazakii* strains such as strain 6 (ST4) and 680 (ST8) were killed rapidly following uptake, proving the killing ability of microglia. As highlighted before, strain 6 is avirulent in the sense that it was serum sensitive, unable to produce iron siderophore,

and low in cytotoxicity and invasiveness for Caco-2, HBMEC, and HMGC cell lines and that might explain its low survival rates.

A group of genes were found to confer phagocytosis survival in bacteria. PhoP and PhoQ belong to a large family of two-component regulatory systems that allow the bacteria to sense and respond to environmental changes by altering gene expression. Phosphorylated PhoP binds to a specific promoter to repress or induce the expression of more than 40 genes. PhoQ is an integral membrane protein demonstrating histidine kinase activity that responds to the signals by transferring phosphate to a conserved residue in the amino-terminus of PhoP. It was shown that a single point mutation of *phoQ* resulted in increased PhoP phosphorylation leading to the expression of PhoP-activated genes (Miller *et al.* 1989, Gunn *et al.* 1996, Ernst *et al.* 1999). It was also demonstrated that increase Mg^{2+} and Ca^{2+} concentrations could repress the kinase activity of PhoQ leading to decreased expression of PhoP-activated genes. Thus, the displacement of these divalent cations from PhoQ inside the phagocytic cell environment could lead to expression of PhoP-activated genes (Garcıa Véscovi *et al.* 1996, Garcıa Véscovi *et al.* 1997, Ernst *et al.* 1999).

These activated genes were found to play a major role in *Salmonella* pathogenesis including resistance to host defence cationic antimicrobial peptides and low pH, and macrophage survival. The PhoP-activated genes include high-affinity Mg^{2+} transporter encoded by *mgtB* gene and proteins required for the resistance to host antimicrobial peptides of the polymyxin class encoded by *pmrA/B* genes. The latter genes are involved in the modification of the outer membrane structure by making structural changes in the lipid A of LPS to avoid the attack by the antimicrobial peptides (Ernst *et al.* 1999). Moreover, low pH levels could induce the expression of *pmrA* and *pmrB* genes that regulate the resistance to polymyxin via stimulating the expression of *pmrE*, which is predicted to encode UDP-glucose dehydrogenase (Gunn *et al.* 1998, Ernst *et al.* 1999). Moreover, it was demonstrated by Blanc- Potard and Groisman (1997) that *mgtB* mutants of *S.* Typhimurium showed a low replication

rate inside macrophages compared with the wild type indicating the importance of this transporter in low Mg^{2+} adaptation within macrophages.

In *Y. pestis*, PhoP-regulated genes are required for LPS modification and the adaptation of low Mg^{2+} levels in the phagosome that mediates the survival within macrophages. Moreover, PhoP plays an important role in *Y. pestis* to prevent the delivery of cathepsin D to its vacuole in macrophages (Grabenstein *et al.* 2004, Grabenstein *et al.* 2006). Cathepsin D is an acid-acting macrophage lysosomal protease that provides bacterial killing (Bewley *et al.* 2011). In addition, it was shown that *phoP* mutant of *Y. pestis* was unable to grow on Mg^{2+}–depleted solid medium compared with the wild type, indicating the importance of the magnesium transporters that are regulated by PhoP such as MgtB (Snavely *et al.* 1991, Oyston *et al.* 2000). Furthermore, it was reported by Yamamoto *et al.* (1996) that GsrA protein is important in the intracellular survival within macrophages by *Y. enterocolitica*. It serves as a stress protein that degrades the abnormal stress peptides before their accumulation to toxic levels in the periplasmic space. The same study showed that the mutation of the *gsrA* gene resulted in inability of the organism to survive within macrophages that is attributed to the increased sensitivity to oxidative stress.

Therefore, it is proposed that PhoQ of *C. sakazakii* is activated following the oxidative stress, low pH, and Mg^{2+} limitation applied by phagocytic cells as a defensive mechanism, which could lead to the phosphorylation of PhoP. As a consequence, this will activate PhoP-regulated gene expression including *pmrABE* and *mgtB* genes. This allows the organism to avoid killing by the antimicrobial peptides and acquire Mg^{2+}. Moreover, the presence of *gsrA* and *sodA*, which is responsible for SOD production, could provide a protection against oxidative stress killing by phagocytic cells. This might afford a mechanism, which *C. sakazakii* strains could utilise to survive phagocytosis.

It was shown previously in this research that strain 6 (ST4), which is negative for the *mgtB* gene, was taken up and killed rapidly by macrophages and microglial cells indicating its high susceptibility for

phagocytosis (Fig.3.23 and Fig.3.25). This indicates that the presence of *mgtB* gene might be required for the survival within phagocytic cells. However, strains 558 (ST4) and 680 (ST8) that are positive for the *mgtB* gene also displayed low survival and multiplication inside macrophages and microglial cells in spite of the presence of all tested genes required for phagocytosis survival. Moreover, even though ST4 strains 6 and 558 in addition to strain 680 (ST8) showed the presence of the phagocytosis survival-associated genes in their genomes, apart from *mgtB* gene in strain 6, these genes might not be functional in those strains or other genes are also involved in the phagocytosis survival. In addition, although strains 658 (ST1) and 696 (ST12) were positive for all these genes, they were able to multiply within macrophages but not inside microglial cells (Fig.3.23 and Fig.3.25). This proposes that the mechanism of macrophage killing might differ from the one for microglial cells and requires the expression of different genes. All other strains that encode the tested phagocytosis-related genes were able to survive and multiply within macrophages and microglial cells, indicating their ability to avoid phagocytosis in addition to long-term survival within these cells and this might increase their virulence (Fig.3.23 and Fig.3.25). The previous mechanisms need further investigations especially at the functional genomic levels.

A study by Townsend *et al.* (2007b) has tried to link the expression of SOD by *Cronobacter* strains to the survival within human macrophages. Although some of SOD expression results correlated with the survival results, on the other hand, strain NTU3, which is *Cronobacter muytjensii* (ATCC 51329), showed high SOD activity but low survival. This, however, can not rule out the function of *sodA*, as this gene shows a degree of expression variability according to the same study.

This chapter discussed some aspects regarding *C. sakazakii* virulence that support the infection and the pathogenic process. Although it did not investigate all virulence factors, the ones that were examined gave a part of the information needed to understand the strategies whereby the organism could overcome the host barriers. The genome of *C. sakazakii*

encodes a group of virulence genes. Possessing these genes is vital for the organism to survive within the host. The genome of *C. sakazakii* needs functional genomic studies that could help in finding the functional genes and study their role in infection and pathogenesis in the laboratory.

The invasion potential is important for *C. sakazakii* to establish a successful infection. This step leads to the translocation of the bacterium to the underlying tissues causing more damage and allowing the organism to reach the blood flow. Most *C. sakazakii* strains were serum resistance and therefore they are able to survive in blood by avoiding serum-mediated killing. Moreover, the organism is able to survive and multiply inside macrophages, which allow it to cause bacteraemia by intracellular reproduction and the dissemination throughout the body. Furthermore, the bacteraemia could provide a suitable increased intracranial pressure for the pathogen to cross the BBB by the translocation through the brain endothelium and resist phagocytosis by microglia in the brain parenchyma. This, however, needs further clarification by exploring the host response and its role in the outcome.

Chapter 4: Host Response

4.1. Introduction

The Gram-negative bacterium *C. sakazakii* has been linked to a number of confirmed meningitis cases worldwide and has been known to cause sepsis and meningitis among neonates in particular, in addition to NEC and bacteraemia (Joker *et al.* 1965, Lai 2001, Joseph and Forsythe 2011). Contaminated PIF was associated with neonatal outbreaks, which resulted in severe clinical outcomes (Muytjens *et al.* 1983, Biering *et al.* 1989, Himelright *et al.* 2002, Townsend *et al.* 2007b). Urmenyi and Franklin (1961) reported the first two *Cronobacter* spp. neonatal meningitis cases, and the organism was then still classified as pigmented strains of *Enterobacter cloacae*. A study by Muytjens *et al.* (1983) showed that newborns with *Cronobacter* spp. meningitis have a poor prognosis, and the fatality rate in that study was 80% (Muytjens *et al.* 1983). Another study reported 3 cases of meningitis caused by the same organism which resulted in one death and brain damage in the two survivors (Biering *et al.* 1989).

The case-fatality rate of neonates due to *C. sakazakii* infection was reported to be 50% with half of the patients dying within one week of diagnosis. *C. sakazakii*-induced meningitis has a tendency to result in brain infarction and severe neurologic impairment (Jiménez and Giménez 1982, Howkins *et al.* 1991, Lai 2001). Despite the growing reported incidence of neonatal meningitis caused by *C. sakazakii*, there is still incomplete understanding of the pathogenesis, pathophysiology, and the role of the host response in this disease. Factors such as NO secretion, apoptosis induction, and cytokine production might contribute, as part of the host response, in the pathogenesis leading to severe outcomes (Iben and Rodriguez 2011, Barichello *et al.* 2013). Although most of the studies were focusing on the role of *Cronobacter* isolates in necrotising enterocolitis (NEC) and the impact of the host response on the onset of the disease, there was insufficient knowledge about *C. sakazakii*-induced meningitis and the host response towards the infection despite the growing numbers of the severe cases of meningitis and brain infections.

Nitric oxide (NO) is a small free-radical gas that promptly diffuses in cells and cell membranes where it interacts with molecular targets (Gross and Wolin 1995). It is synthesised after enzyme activation by NO synthases, and the toxic molecule of NO is synthesised primarily by inducible nitric oxide synthase (iNOS) that is found in the fully activated mammalian cells such as macrophages and endothelial cells. The cytotoxicity is usually linked to NO produced by iNOS and not to the products of the other NO synthases (Kröncke *et al.* 1997, Aktan 2004). The expression of iNOS during inflammation increases the production of NO significantly (Hunter *et al.* 2009). NO was found to play a major role in pathophysiological conditions including neurodegeneration and chronic inflammation. Moreover, it is a cytotoxic effector molecule that displays cytotoxic activity *in vivo*. It is utilised by the immune system to fight invading pathogens, it can however be toxic to host tissues if overproduced and might contribute to cell damage or death (Gross and Wolin 1995, Kröncke *et al.* 1997, Mayer and Hemmens 1997, Aktan 2004).

It was reported that *Cronobacter* was able to induce iNOS expression in human and mice intestinal cells that would lead to increased NO production, which in turn triggers apoptosis that might alter the tight junctions (Hunter *et al.* 2009, Emami *et al.* 2012, Liu *et al.* 2012b). To the author's knowledge, none of the published studies has examined the ability of *C. sakazakii* strain to induce iNOS and NO production in human brain micovascular endothelial cells (HBMEC) despite the fact that iNOS can be produced by these cells leading to increased NO production that cause cytotoxicity. Therefore, it is important to assess iNOS induction, which might contribute to alteration of the tight junctions of this cell line besides triggering apoptosis.

Apoptosis is programmed cell death that is characterised by morphological alterations and a set of cellular changes including cell shrinkage, chromatin condensation, nuclear DNA cleavage, and membrane blebbing (Cohen *et al.* 1992, Dimmeler and Zeiher 1997). Inducing cell death via apoptosis in human cell lines might collaborate in the permeability of these cell lines by contributing to the disruption of the tight junctions. It

has a major impact on the integrity of the gut barrier, as it is a characteristic feature of NEC that will lead to the translocation of the invading microorganism through the permeable infected layer (Hunter *et al.* 2008, Liu *et al.* 2012b, Hunter and Bean 2013). According to Liu *et al.* (2012b) *C. sakazakii* strains were able to induce apoptosis of human Caco-2 cells and rat IEC-6 cells. Due to the lack of research concerning *C. sakazakii*-induced HBMEC cell line apoptosis, it is important to assess the ability of *C. sakazakii* to trigger this response. HBMEC monolayers were used to represent the brain endothelium *in vitro* and inducing apoptosis in these cells might lead to the disruption of the integrity of the monolayer, which *in vivo* could lead to the migration of the organism towards the human brain.

C. sakazakii infection triggers a group of immune responses, such as cytokine and chemokine production. Different inflammatory molecules are involved in the pathogenesis including TNF-α and IL-6 (Hunter *et al.* 2008). A study by Townsend *et al.* (2007b) showed that *C. sakazakii* strains were able to stimulate the human macrophage cell line U937 to produce TNF-α, IL-6, and IL-10, however it did not suggest the role of these cytokines in infection. It was reported by Cruz-Córdova *et al.* (2012) that flagella of *C. sakazakii* play an important role in triggering the host immune response and the activation of cytokine production such as IL-8 and TNF-α by macrophage and HEK293 cell lines. Although all these studies showed the ability of the organism to initiate cytokine production, they however did not propose any mechanism of action of the detected cytokines. Moreover, most *Cronobacter* cytokine studies were applied on animal cells or tissues and did not use human cell lines that are more relevant in increasing knowledge regarding human infections.

As mentioned previously, the vast majority of the available host response studies have only investigated the role of the *C. sakazakii* infection in gut damage especially in NEC, and most of them used animal models and animal cell lines. To date there are no publications that have used human brain cells and indeed the human brain microvascular endothelial cells (HBMEC) cell line (as a component of the BBB), and human microglial

cells (HMGC) cell line (as a part of innate immunity of the brain) to investigate the ability of *C. sakazakii* strains to induce iNOS production, apoptosis, and cytokine secretion by these cells. Moreover, microglia have a crucial role in the clearance of infection as a part of innate immunity (Polazzi and Monti 2010). Thus, understanding the role of these responses and molecules can be useful to evaluate the pathogenic process. The functions of the cytokines produced by endothelial cells and microglia in *C. sakazakii*-induced meningitis could help to clarify the mechanism of the brain infection and the factors that affect the host barriers including cell line permeability and chemoattraction. Therefore, this current research aims to study the host response to *C. sakazakii* brain infection *in vitro* and the impact of the infection on BBB. Moreover, it will assess the potential role of this response in pathogenesis and whether it contributes in the disease or not.

4.2. Materials and Methods overview

In this chapter, *C. sakazakii* strains were examined for their ability to induce iNOS production by HBMEC cells using a human total iNOS immunoassay. Moreover, the strains were investigated for their ability to induce apoptosis to HBMEC and HMGC cell lines using fluorescence-tagged stains. In addition, cytokine production by these cell lines as a response of *C. sakazakii* infection was tested by Bio-Plex® ELISA. The methods, media, cell line maintenance, and culture preparation were described previously in Chapter 2 (Materials and Methods) Section 2.6.

For statistical analysis, data were assessed for normality using Kolmogorov-Smirnov test and normality histograms. The normally distributed data were analysed using the parametric One-way Analysis of Variance test (ANOVA) with Tukey's post-hoc test, and were expressed as mean values and the standard error of mean (Mean±SEM). Tukey's post-hoc analysis was performed to compare the significance of the means of every *C. sakazakii* strain in relation to other strains as pairwise comparisons. A *P*-value of <0.05 was considered statistically significant.

4.3. Results

4.3.1. Human total iNOS immunoassay

The production of iNOS leads to elevated NO levels, which increases the cell line permeability, thus helping the bacteria to migrate into the site of infection. The levels of iNOS were detected using human total iNOS immunoassay. Most of the CC4 strains showed the capacity to induce elevated levels of iNOS, while strains 553 and 1465 generated modest levels. However, strains 6 and 558 showed the lowest induction levels of iNOS. For the non-CC4, strains 1249 (ST31), 555 (ST1), 1019 (ST1), 1241 (ST1), 680 (ST8), 520 (ST12), and 696 displayed high iNOS induction levels. Nonetheless, strains 12 (ST1), 658 (ST1), 1 (ST8), 5 (ST8), 580 (ST18) and the negative control (*E. coli* K12) (control wells with no primary antibody- no bacteria used for calculations- materials & methods) exhibited the lowest levels of iNOS production (Fig.4.1).

The strains that showed low iNOS induction levels such as strain 6 (ST4) and 558 (ST4) were not able to translocate through HBMEC cell monolayers or demonstrated declined translocation levels e.g. strain 658 (ST1; Table.4.1-2 and Fig.3.20). These results support the role of NO in translocation, as the high levels of iNOS leads to elevated NO levels (Hunter *et al.* 2009), which might contribute in the translocation assay by causing cell line permeability. However, strains 553 (ST4), 557 (ST4), 1249 (ST31), 555 (ST1), 1019 (ST1), 1241 (ST1), 680 (ST8), and 520 (ST12) were high in iNOS induction and showed low translocation ability (see Fig.3.20, Fig.4.1, and Table.4.1). These anomalies suggest that these strains might not be NO-dependent isolates in terms of translocation, and the translocated strains might use other mechanisms such as apoptosis induction or cytokine-dependent translocation in which the bacterium triggers the cell line to produce cytokines that could cause cell line permeability.
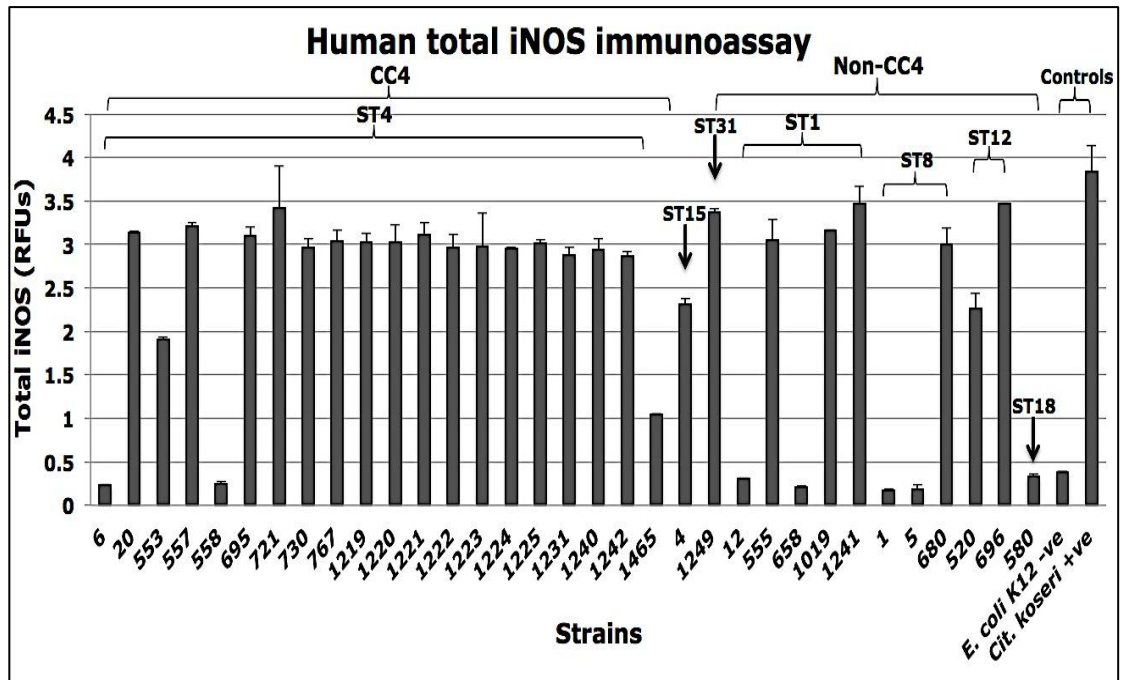
Fig.4.1. *C. sakazakii* induced iNOS expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 96-well microplates and infected with *C. sakazakii* strains for 3 hours. After fixation of cells in the wells, iNOS levels were determined using human total iNOS cell-based ELISA standardised using GADPH. Values represent mean±range of normalized duplicate determinations.

RFUs: relative fluorescence units.

Table.4.1: Summary of HBMEC translocation and iNOS induction results.

Strain	ST	HBMEC invasion[1]	HBMEC translocation[2]	iNOS induction[3]	Source	Clinical presentation
695	4	Moderate	High	High	Trachea	Fatal NEC II
767	4	High	High	High	Trachea	Fatal meningitis
696	12	High	High	High	Faeces	NEC II
20	4	Moderate	High	High	Clinical	Unknown
1221	4	High	High	High	CSF	Meningitis
1240	4	High	High	High	CSF	Fatal meningitis
1249	31	Moderate	Low	High	Clinical	Fatal isolate
1242	4	High	Low	High	Brain	Fatal meningitis
658	1	Low	Low	Low	NIF	Unknown
680	8	Low	Low	High	CSF	Unknown
558	4	Low	None	Low	Clinical	Unknown
6	4	No	None	Low	Clinical	Unknown
553	4	Low	None	Moderate	Clinical	Unknown
557	4	Low	Low	High	Clinical	Unknown
721	4	Moderate	Moderate	High	CSF	Unknown
730	4	Low	Low	High	Clinical	NEC I
1219	4	Moderate	High	High	CSF	Fatal meningitis
1220	4	Moderate	High	High	CSF	Brain abscess
1222	4	Moderate	High	High	Blood	Fever
1223	4	Moderate	High	High	Blood	Unknown
1224	4	Moderate	Moderate	High	Blood	Fever
1225	4	Moderate	Moderate	High	Blood	Fatal meningitis
1231	4	Moderate	Moderate	High	Faeces	Meningitis
1465	4	Low	None	Low	PIF	Unknown
4	15[a]	Low	None	High	Clinical	Unknown
12	1	Low	None	Low	Faeces	Unknown
555	1	Low	Low	High	Clinical	Unknown
1019	1	Low	Low	High	CSF	Fatal meningitis
1241	1	Moderate	Low	High	Blood	Unknown
1	8	Low	None	Low	Throat	Unknown
5	8	Low	None	Low	Clinical	Unknown
520	12	Low	Low	High	Clinical	Unknown
580	18	Low	Low	Low	Clinical	Unknown

ST: sequence type. **[a]:** part of clonal complex 4 (CC4).
CSF: cerebrospinal fluid **PIF:** powdered infant formula. **ND:** not done.
NIF: non-infant formula. **NEC:** necrotising enterocolitis.
[1]: Fig.3.14 Chapter.3. **[2]:** Fig.3.20 Chapter.3. **[3]:** Fig.4.1.

4.3.2. Apoptosis marker detection

C. sakazakii strains were tested for their ability to induce apoptosis in HBMEC and human microglial (HMGC) cell lines over 3 hours of incubation. Fluorescence stains for caspase-3 and annexin V were applied to test the infected cells. The majority of *C. sakazakii* strains that were used showed the ability to trigger apoptosis in the infected cell lines and high fluorescence signals were detected in all samples for caspase-3 and annexin V. These high signals indicated high concentrations of the two tested apoptotic markers. The apoptotic cells appeared to be shrunken and rounded (Fig.4.2). Cells incubated with ST4 strains 767 and 1240, which showed high levels of translocation through HBMEC cells and persistence and multiplication in HMGC (Fig.3.20, Fig.3.25, and Table 4.2), displayed more intense signals, while signals generated in cells incubated with the other strains were slightly lower (Fig.4.3.). On the other hand strains 6 (ST4), 658 (ST1), and 680 (ST8) demonstrated low fluorescence signals indicating low induction levels of caspase-3 and annexin V. These strains did not translocate (6) through HBMEC cell line or showed low translocation (658 and 680) and were killed by microglia (Fig.3.20, Fig.3.25, and Table.4.2). Although strain 658 (ST1) generated a low fluorescence intensity, the signals obtained from the cells infected with this strain were higher than those generated by strains causing low levels of apoptosis (Fig.4.3). Inducing apoptosis is potentially important for the organism to overcome host physical barriers by causing cell line permeability. Moreover, it is a sign to indicate that the host response to *C. sakazakii* infection likely has a role in pathogenesis by triggering cell death that could contribute in more damage to the host.
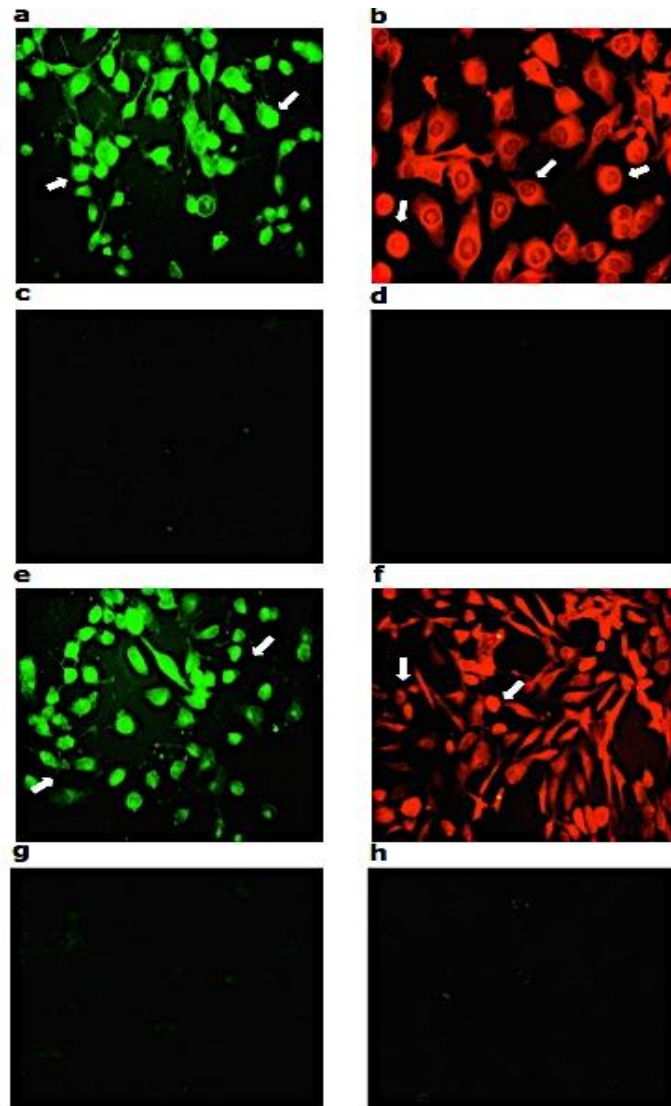
Fig.4.2. Fluorescence microscope images of *C. sakazakii*-infected HBMEC and HMGC cell lines. **(a and b)** HBMEC cell line infected with *C. sakazakii* strain 767 (ST4) stained by caspase-3 (a) and annexin V (b) fluorescence stains, showing intense green (a) and red (b) signals, and the cells that have morphological changes indicated by the arrows. **(c and d)** Negative control images control of induced cells without adding the primary antibody. **(e and f)** HMGC cell line infected with *C. sakazakii* strain 1240 (ST4) stained by caspase-3 (c) and annexin V (d) fluorescence stains, showing intense green (c) and red (d) signals, and the cells that have morphological changes indicated by the arrows. **(g and h)** Negative control images control of induced cells without adding the primary antibody.
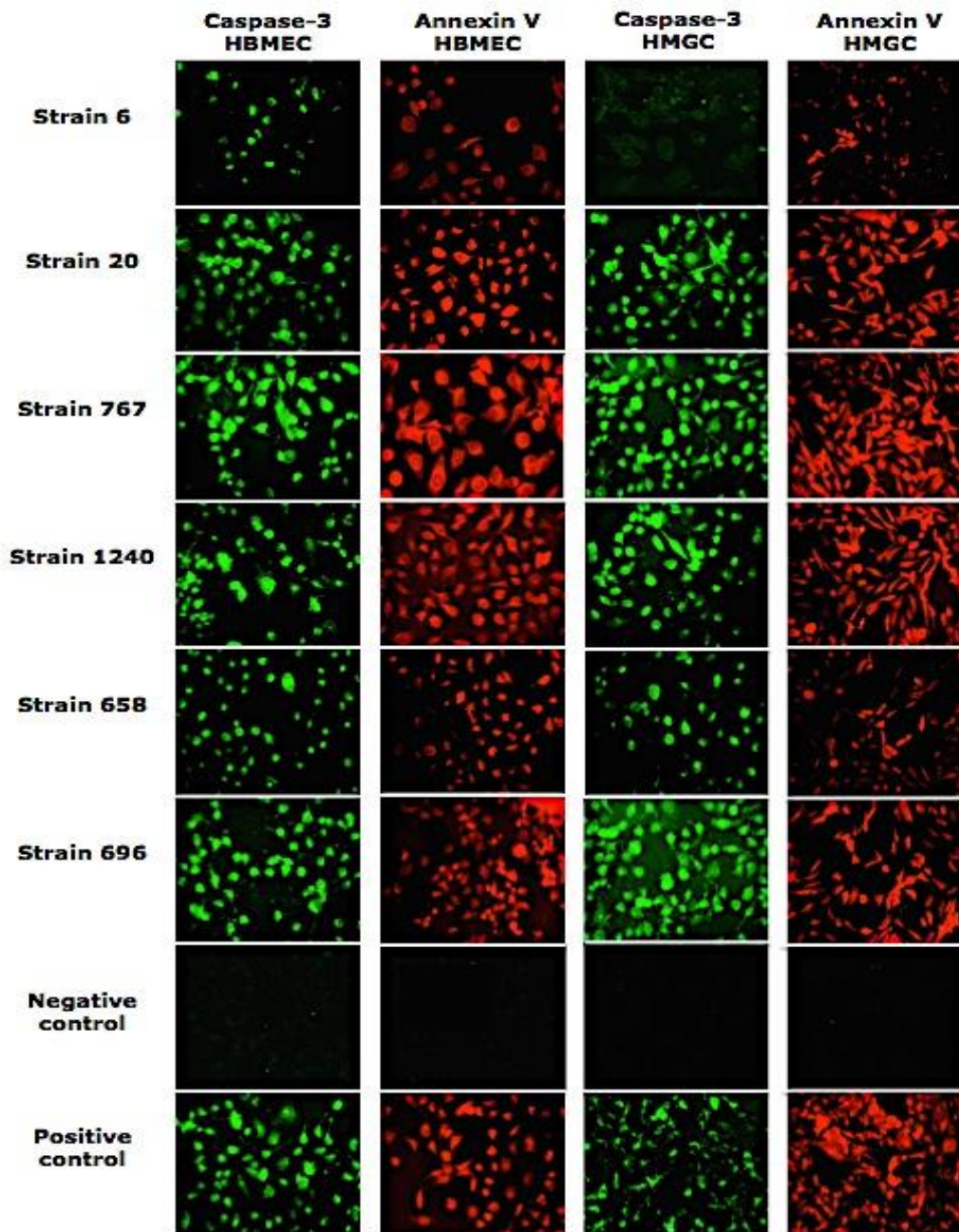
Fig.4.3. Fluorescence microscopy analysis of apoptosis profile of *C. sakazakii*-infected HBMEC and HMGC cell lines. *C. sakazakii* strains were tested for their ability to induce caspase-3 and annexin V production in these cell lines. The strains were taken as representatives to demonstrate apoptosis induction by *C. sakazakii* infection. HBMEC and HMGC cell lines showed low fluorescence signals when infected with strains 6 (ST4) and 658 (ST1), indicating their low ability to induce apoptosis. On the other hand, the other strains showed high fluorescence signals demonstrating high caspase-3 and annexin V concentrations in those samples as the higher signal the higher concentration and *vice versa*. The negative control images are for induced cells stained with secondary antibody with no primary antibody added. The Positive control images are for induced cells using staurosporine.

4.3.3. Cytokine production detection and profiling

Cytokine production assay was conducted using *C. sakazakii*-infected HBMEC and HMGC cell line supernatants over 5 hours of incubation based on results of the translocation assay, as the strains were able to translocate after 5 hours of infection. The kit used was designed to detect 10 human cytokines including GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α.

C. sakazakii strains were able to trigger the secretion of seven cytokines; IL-1β, IL-6, GM-CSF, TNF-α, IL-4, IL-8, and IL-10. Six *C. sakazakii* strains (CC4 and non-CC4) were included in this experiment based on several criteria obtained from the previous experimental results and the clinical history of these strains such as the site of isolation and the nature of infection, the invasiveness and translocation ability, and phagocytosis survival. These strains include low invasion and translocation, and plasmid-less strain from ST4 (strain 6) and two invasive and translocated strains, 767 and 1240, from the same ST that was linked to most of the meningitis cases among *C. sakazakii* STs (Joseph and Forsythe 2011). Additionally, strain 658 (ST1), which was isolated from non-infant formula and was the first sequenced strain, was included to the test strains. Moreover, strain 696 (ST12), which was linked to NECII infection in the French NICU outbreak in 1994. This will permit a rational comparison of *C. sakazakii* strains from different STs and isolation sources. Moreover, this could add further information about the behaviour of the strains that were able to cause meningitis and if it is different from those that did not cause meningitis. In addition, this assay will evaluate the response of HBMEC to *C. sakazakii* infection in the absence of flagella by using the non-motile *fliC* - negative strain 680. *Cit. koseri* is a Gram-negative bacterium that was associated with several cases of neonatal meningitis (Ribeiro *et al.* 1976, Rose 1979, Doran 1999, Pollara *et al.* 2011). This organism was the positive control for the brain cell lines (endothelial cells and microglia), thus it was included to investigate if responses of these cell lines to *Cit. koseri* and *C. sakazakii* were similar.

4.3.3.1. Cytokine production of *C. sakazakii*-induced HBMEC cells

Since HBMEC cells represent the first layer of the BBB that provides the protection against any invading microorganisms coming from the blood, cytokine secretion from these cells was assessed via Bio-Plex$^{®}$ ELISA following *C. sakazakii* inoculation.

C. sakazakii strains showed the capacity to induce IL-1β secretion and strains 767 (ST4), 1240 (ST4), and 680 (ST8) caused high levels of IL-1β production (test sensitivity <5 pg/ml). Strains 658 (ST1), and 696 (ST12) induced significantly higher concentrations of IL-1β compared to the other strains tested (>50 pg/ml; $P<0.05$), while *Cit. koseri* induced the highest level of IL-1β ($P<0.001$). In contrast, *C. sakazakii* strain 6 (ST) the non-invasive and non-translocating strain induced a low level of IL-1β secretion (Fig.4.4).

With regard to TNF-α, different induction trends were recorded among *C. sakazakii* strains (test sensitivity <1 pg/ml). Strain 767 (ST4) generated the most robust response among strains (>70 pg/ml; $P<0.001$), and strain 1240 (ST4) also showed significant levels of TNF-α induction compared to the other strains tested (>23 pg/ml; $P<0.05$). Strains 658 (ST4), 696 (ST12), and *Cit. koseri* showed lower levels of TNF-α induction, while strains 6 (ST4) and 680 (ST8) caused the lowest TNF-α production (<10 pg/ml; Fig.4.5).
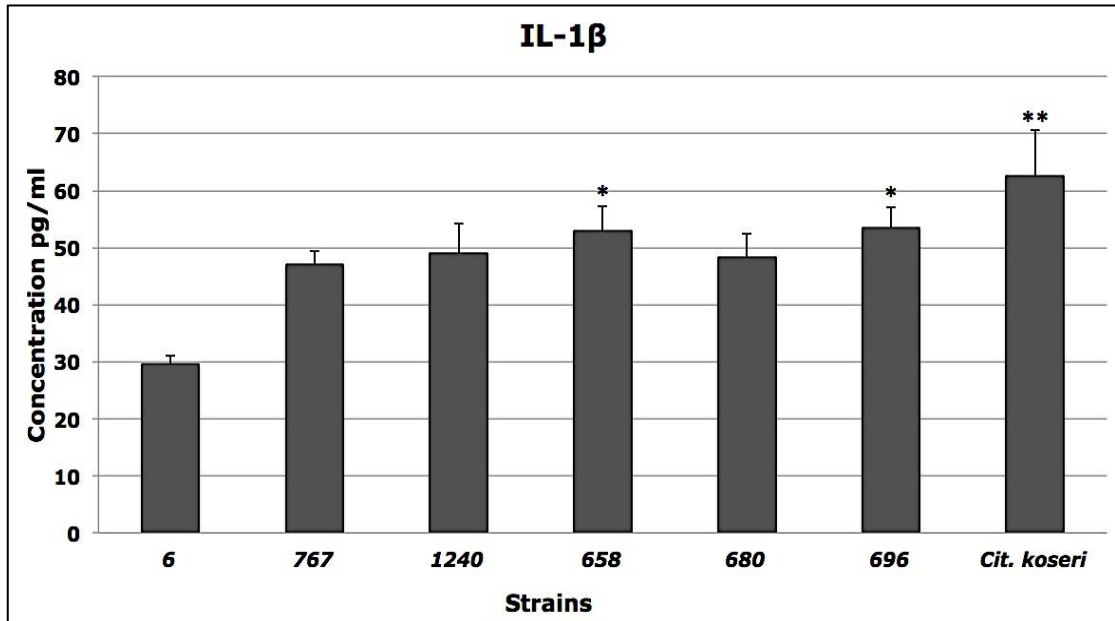
Fig.4.4. *C. sakazakii* induced IL-1β expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-1β levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-1β production between the strains in this experiment are denoted with asterisks (*$P<0.05$, **$P<0.001$; ANOVA).

Fig.4.5. *C. sakazakii* induced TNF-α expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, TNF-α levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of TNF-α production between the strains in this experiment are denoted with asterisks (*$P<0.05$, **$P<0.001$; ANOVA).

C. sakazakii strains generally induced high IL-6 production though for strain 680 (ST8) a much lower response was detected (<450 pg/ml; test sensitivity <1 pg/ml). Strain 6 (ST4) displayed moderate induction, whereas IL-6 levels induced by strain 767 (ST4) and *Cit. koseri* were higher (>1200 pg/ml). In addition, strain 696 (ST12) showed a significantly elevated level of IL-6 induction ($P<0.05$), while strains 1240 (ST4) and 658 (ST1) caused production of the highest levels of IL-6 (>1500 pg/ml) ($P<0.001$; Fig.4.6).

IL-8 induction levels were the highest among all secreted cytokines tested in this experiment showing concentrations >3500 pg/ml (test sensitivity <5 pg/ml). Strain 696 (ST12), and *Cit. koseri* induced significantly increased levels of IL-8 after 5 hours of incubation ($P<0.05$), whereas ST4 strains 767 and 1240 induced the highest levels detected (>3900 pg/ml; $P<0.01$). Strains 6 (ST4), 658 (ST1), and 680 (ST8) showed the lowest levels of IL-8 induction (Fig.4.7).
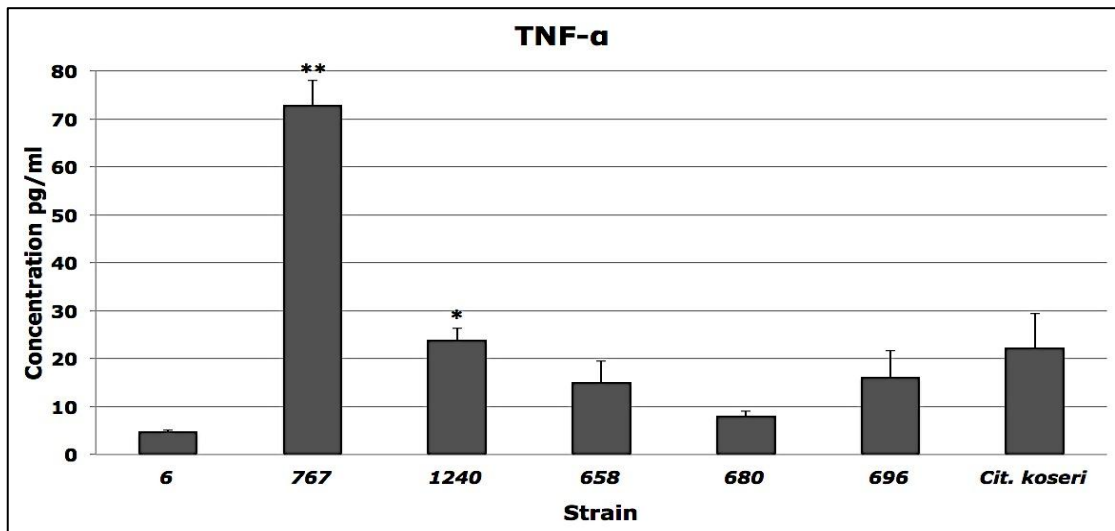

Fig.4.6. *C. sakazakii* induced IL-6 expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-6 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-6 production between the strains in this experiment are denoted with asterisks (*$P<0.05$, **$P<0.001$; ANOVA).

Fig.4.7. *C. sakazakii* induced IL-8 expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-8 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-8 production between the strains in this experiment are denoted with asterisks (*$P<0.05$, **$P<0.01$; ANOVA).

IL-10 was secreted in very low concentrations (<5 pg/ml) when compared to the other cytokines (test sensitivity <1 pg/ml). Strain 658 (ST1) caused a significant increase in induction ($P<0.05$), whereas there was no significant difference in IL-10 induction between the other strains over 5 hours of incubation (Fig.4.8). The vast majority of *C. sakazakii* strains induced significantly elevated levels of IL-4 (>120 pg/ml; $P<0.001$; test sensitivity <1 pg/ml), and strain 680 (ST8) induced the lowest level detected. Strain 6 (ST4) did not show any IL-4 induction after 5 hours, while *Cit. koseri* displayed a significantly high level of IL-4 induction ($P<0.05$; Fig.4.9).
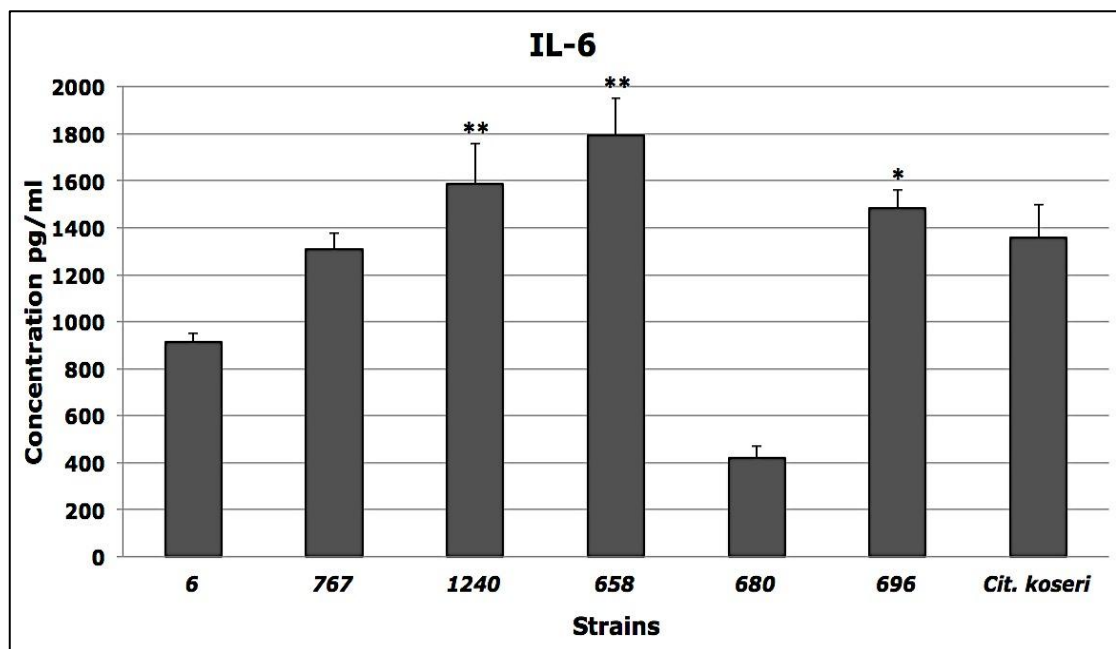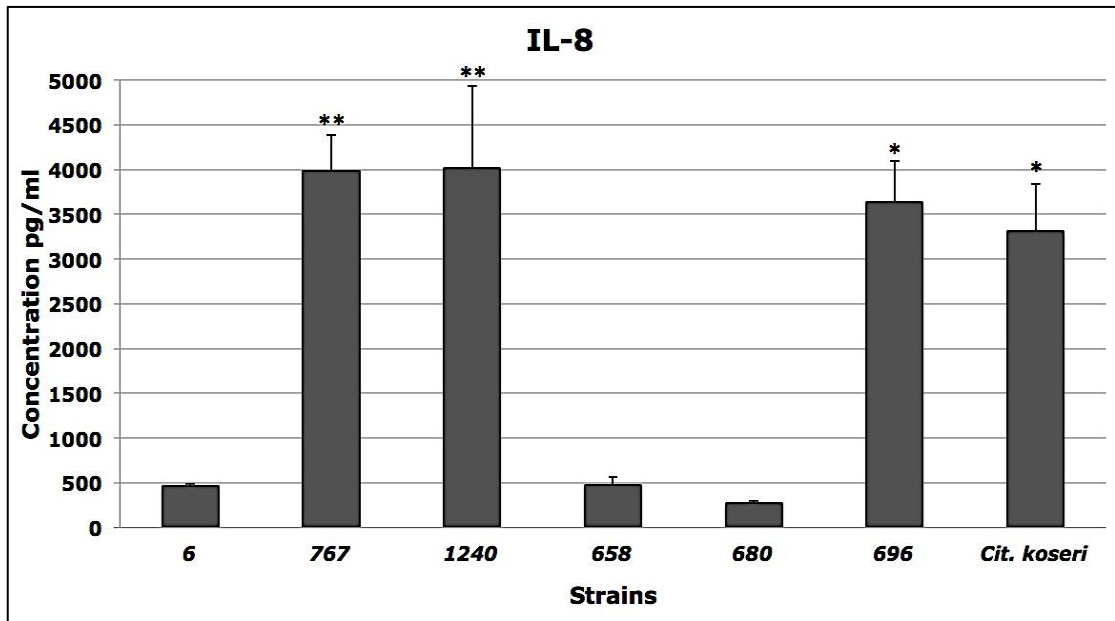
Fig.4.8. *C. sakazakii* induced IL-10 expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-10 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-10 production between the strains in this experiment are denoted with asterisks (*P<0.05; ANOVA).

Fig.4.9. *C. sakazakii* induced IL-4 expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-4 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-4 production between the strains in this experiment are denoted with asterisks (*P<0.05, **P<0.001; ANOVA).

Regarding GM-CSF, it was notable that the induction levels of *C. sakazakii* strains were high except for strain 6 (ST4) (test sensitivity <0.5 pg/ml). ST4 strains 767 and 1250 in addition to 680 (ST8) showed significantly elevated levels ($P<0.05$), while strains 658 (ST1), 696 (ST12) in addition to *Cit. koseri* induced the highest levels (>45 pg/ml; $P<0.001$).
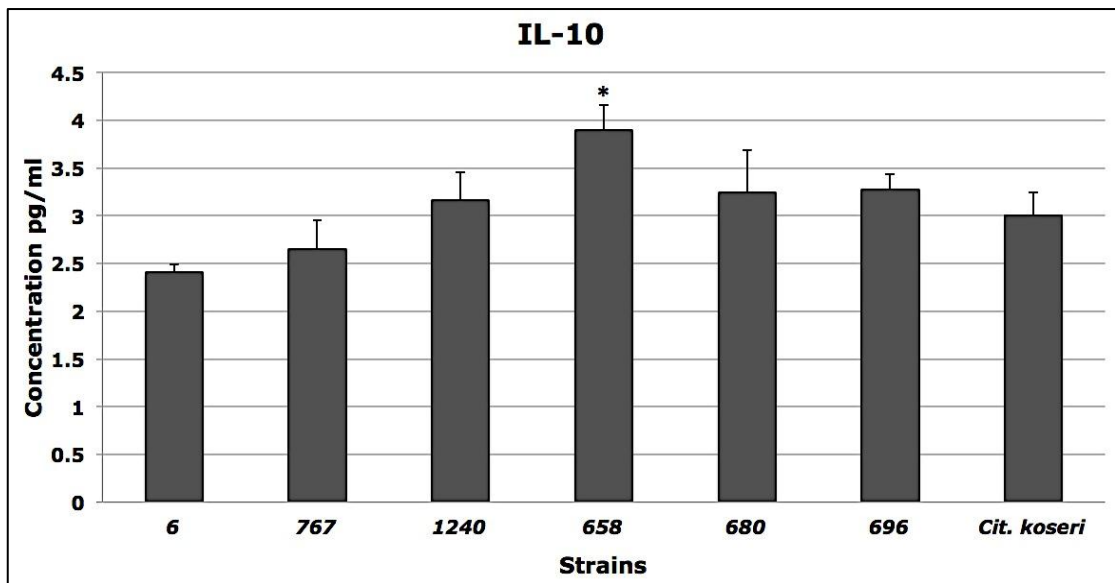

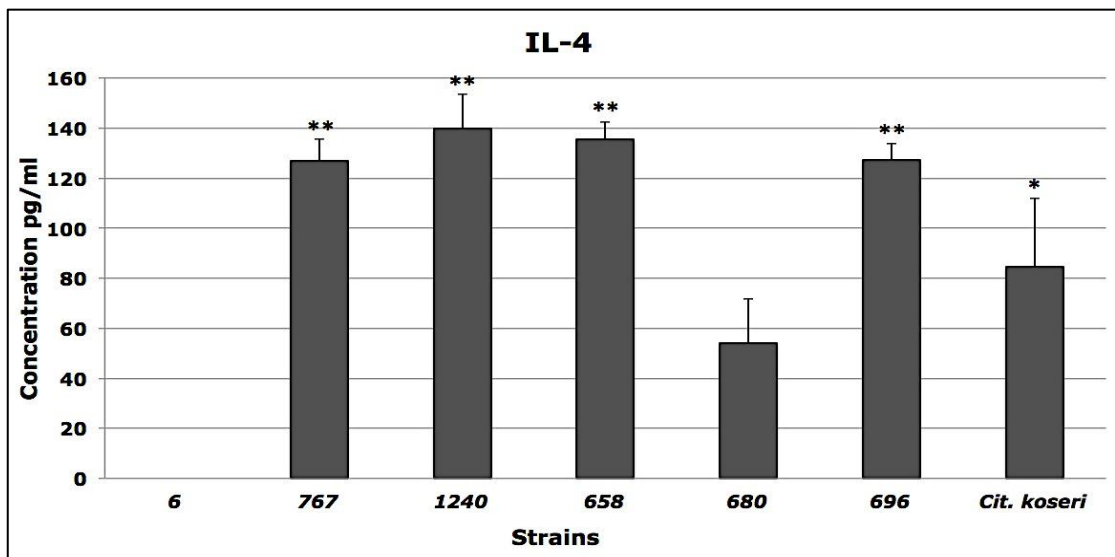**Fig.4.10.** *C. sakazakii* induced GM-CSF expression in human cells. Human brain microvascular endothelial cells (HBMEC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, GM-CSF levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of GM-CSF production between the strains in this experiment are denoted with asterisks (*$P<0.05$, **$P<0.001$; ANOVA).

4.3.3.2. Cytokine production of *C. sakazakii*-induced HMGC cells

Since microglial cells, as brain-resident macrophages, are a part of the innate immune response and responsible for phagocytosis, cytokine secretion from the HMGC cell line was assessed via Bio-Plex® ELISA following *C. sakazakii* infection.

The test strains were able to induce IL-1β production at different levels (test sensitivity <5 pg/ml). ST4 strains 6 and 1240 in addition to 658 (ST1), 696 (ST12), and *Cit. koseri* showed moderate induction levels. However, strain 767 (ST4) induced the highest IL-1β level among the strains tested in this experiment ($P<0.05$; Fig.4.11).

Fig.4.11. *C. sakazakii* induced IL-1β expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-1β levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-1β production between the strains in this experiment are denoted with asterisks (*$P<0.05$; ANOVA).

C. sakazakii strains showed different levels of TNF-α induction. ST4 strains 6 and 1240 induced lowest levels, while strain 696 (ST12) and *Cit. koseri* caused moderate induction. Strain 767 (ST4) caused highest levels of production (>120 pg/ml) ($P<0.05$; Fig.4.12). With regard to IL-6, the HMGC cell line was stimulated to produce high concentrations (>1600 pg/ml) (test sensitivity <1 pg/ml). All *C. sakazakii* strains were able to induce IL-6 responses at high levels except for strain 6 (ST4) that induced a very low concentration when compared to the other strains. Strains 1240 (ST4), 658 (ST1), 696 (ST12) and *Cit. koseri* induced significantly higher levels of IL-6 production ($P<0.01$), whereas strain 767 (ST4) induced the highest level of IL-6 amongst the strains tested in this experiment (>1200 pg/ml) ($P<0.001$; Fig.4.13).

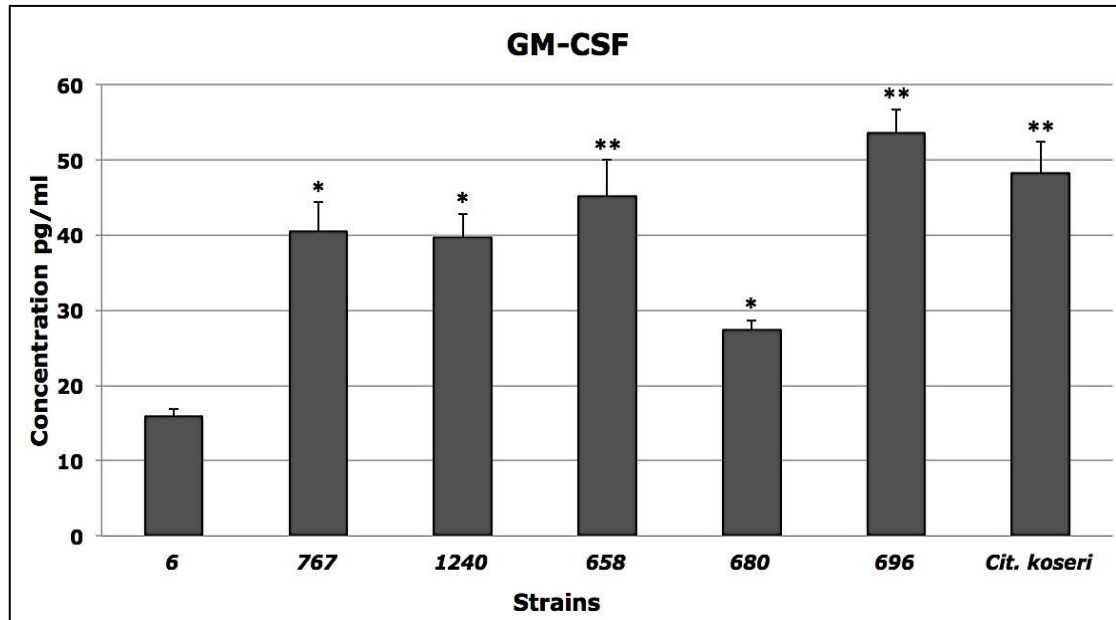**Fig.4.12.** *C. sakazakii* induced TNF-α expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, TNF-α levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of TNF-α production between the strains in this experiment are denoted with asterisks (*$P<0.05$; ANOVA).
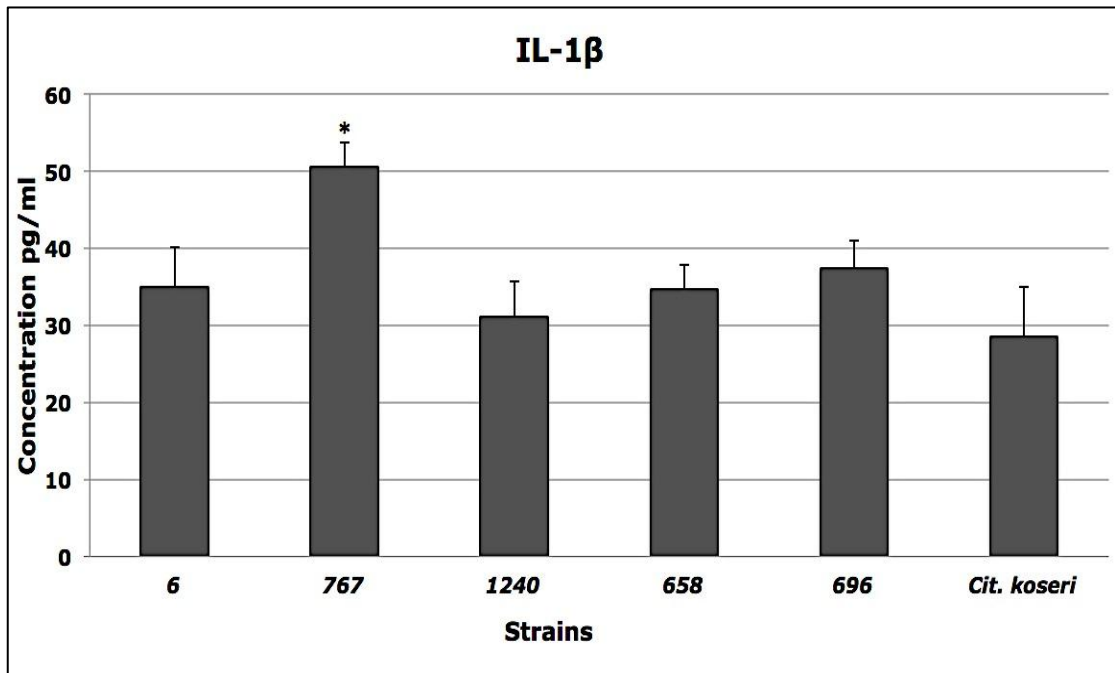

Fig.4.13. *C. sakazakii* induced IL-6 expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-6 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-6 production between the strains in this experiment are denoted with asterisks (*$P<0.01$, **$P<0.001$; ANOVA).

IL-8 was secreted by the HMGC cell line at the highest concentrations among detected cytokines (>3000 pg/ml; test sensitivity <5 pg/ml). Different levels of induction were noticed among *C. sakazakii* strains. Significant IL-8 induction was found with strains 1240 (ST4) and 696 (ST12; *P*<0.05), while strains 767 (ST4), 658 (ST1), and *Cit. koseri* induced the highest levels >2500 pg/ml (*P*<0.001). Strain 6 (ST4) caused the lowest induction <500 pg/ml (Fig.4.14).

Fig.4.14. *C. sakazakii* induced IL-8 expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-8 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments. Significant differences of the mean values of IL-8 production between the strains in this experiment are denoted with asterisks (*P<0.05, **P<0.001; ANOVA).

Among all detected cytokines in this experiment, IL-10 was induced at the lowest levels at concentrations of <3.5 pg/ml (test sensitivity <1 pg/ml). Although different levels of induction were noticed, there were no significant differences in induction among strains. However, ST4 strains 767 and 1240 showed the highest concentrations secreting >3 pg/ml (Fig.4.15).
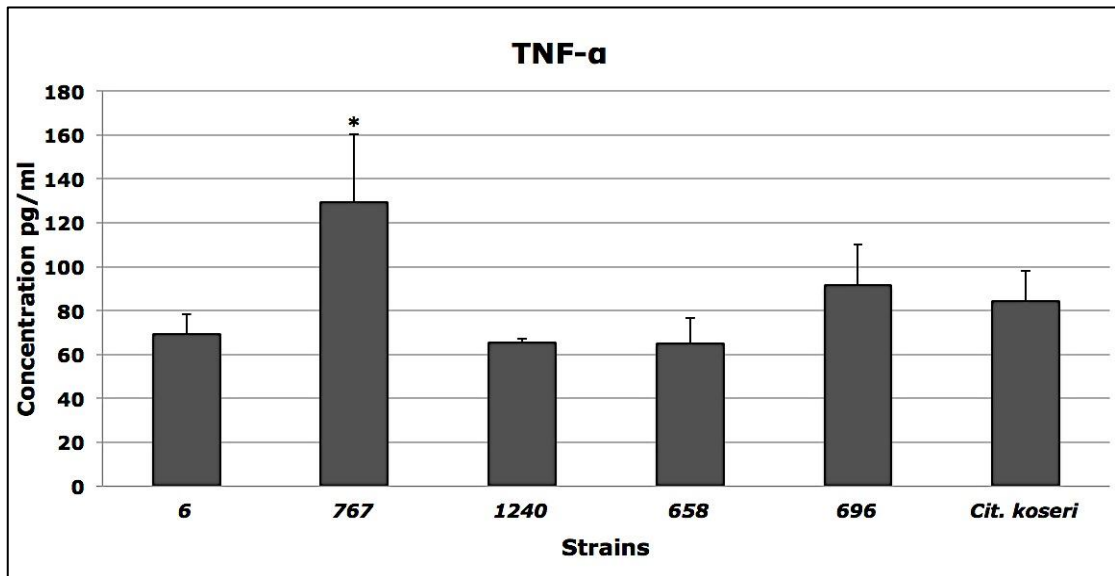
Fig.4.15. *C. sakazakii* induced IL-10 expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-10 levels were determined using Bio-Plex$^{®}$ ELISA. Values represent mean±SEM of triplicate determinations of two experiments.

Most of *C. sakazakii* strains induced IL-4 production (test sensitivity <1 pg/ml). ST4 strains 767 and 1240 in addition to strain 658 (ST1) induced highest levels (>100 pg/ml), while strain 6 ST (4) induced the lowest level. Strain 696 (ST12) and *Cit. koseri* displayed moderate induction levels (Fig.4.16). With regard to GM-CSF, no significant differences were noticed between strains. However, ST4 strains 767 and 1240 and strain 658 (ST1) induced the highest levels. Strain 696 (ST12) and *Cit. koseri* caused moderate stimulation, whereas strain 6 (ST4) showed a low level of induction (Fig.4.17).
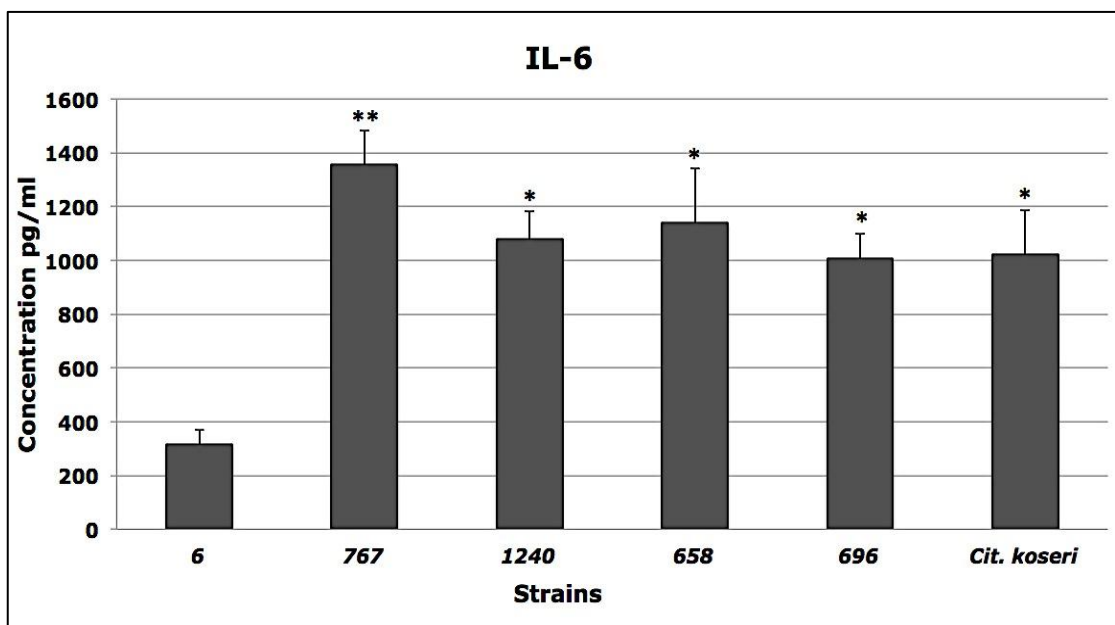
Fig.4.16. *C. sakazakii* induced IL-4 expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, IL-4 levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments.
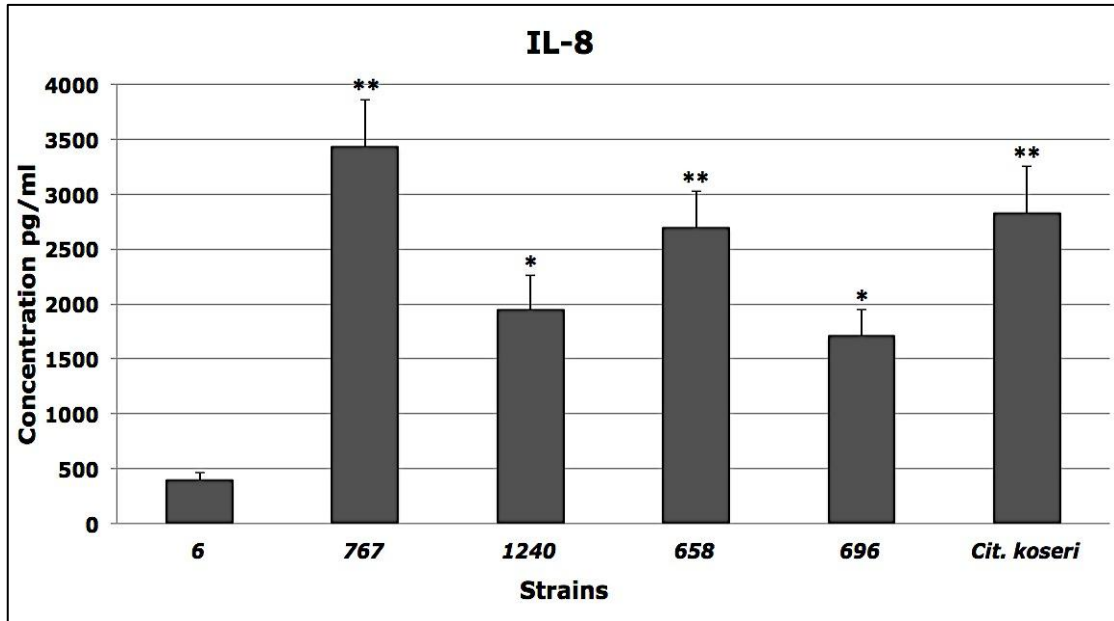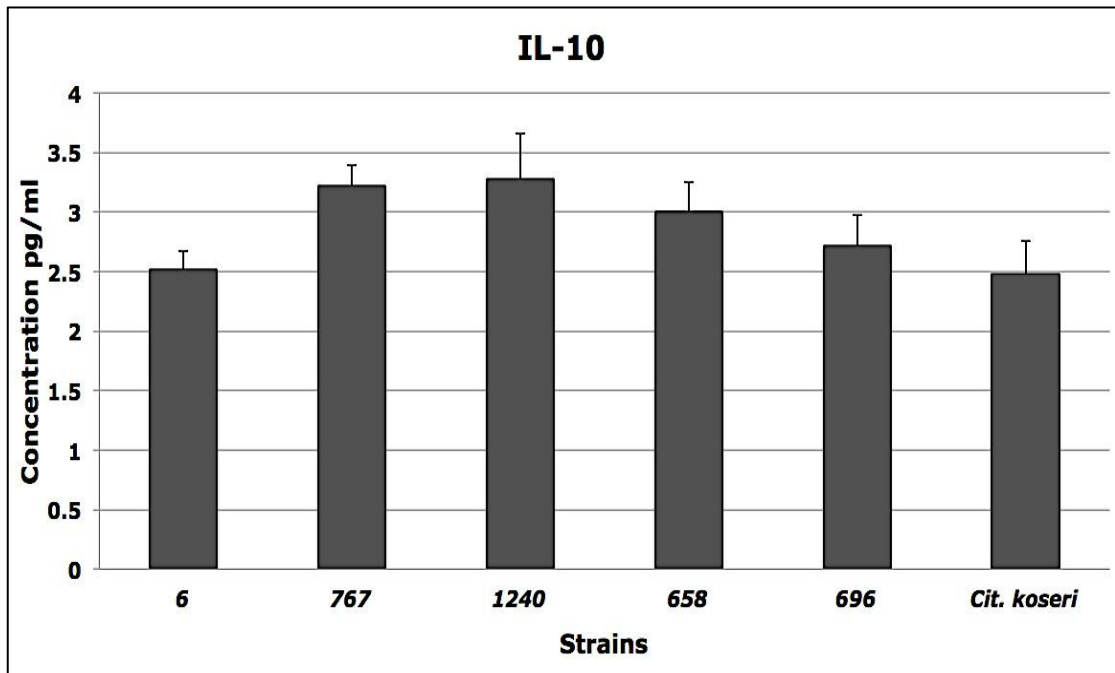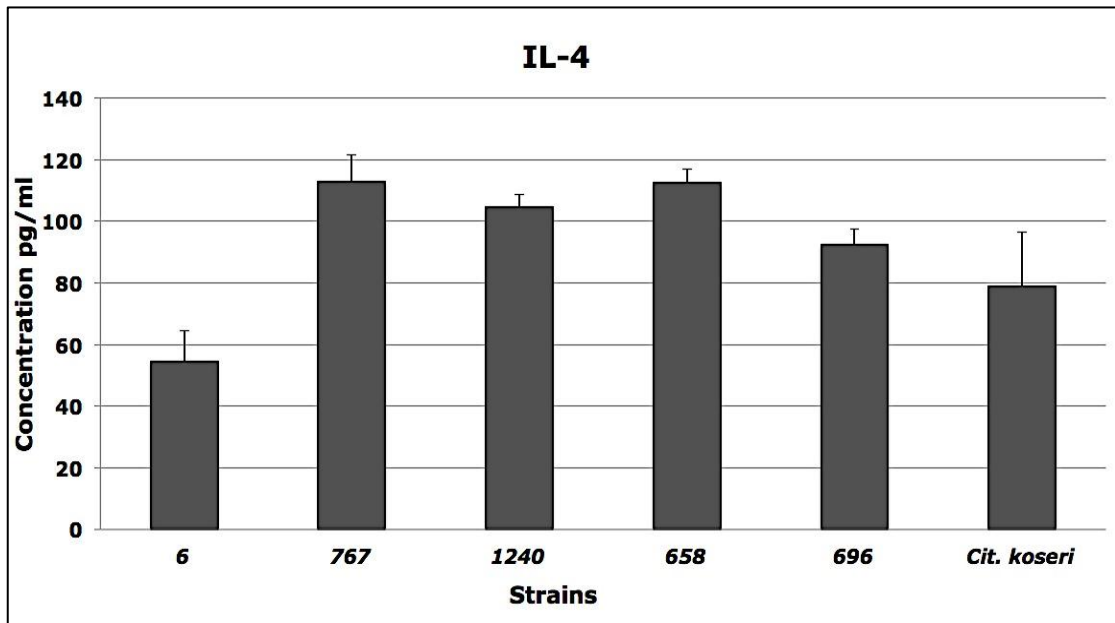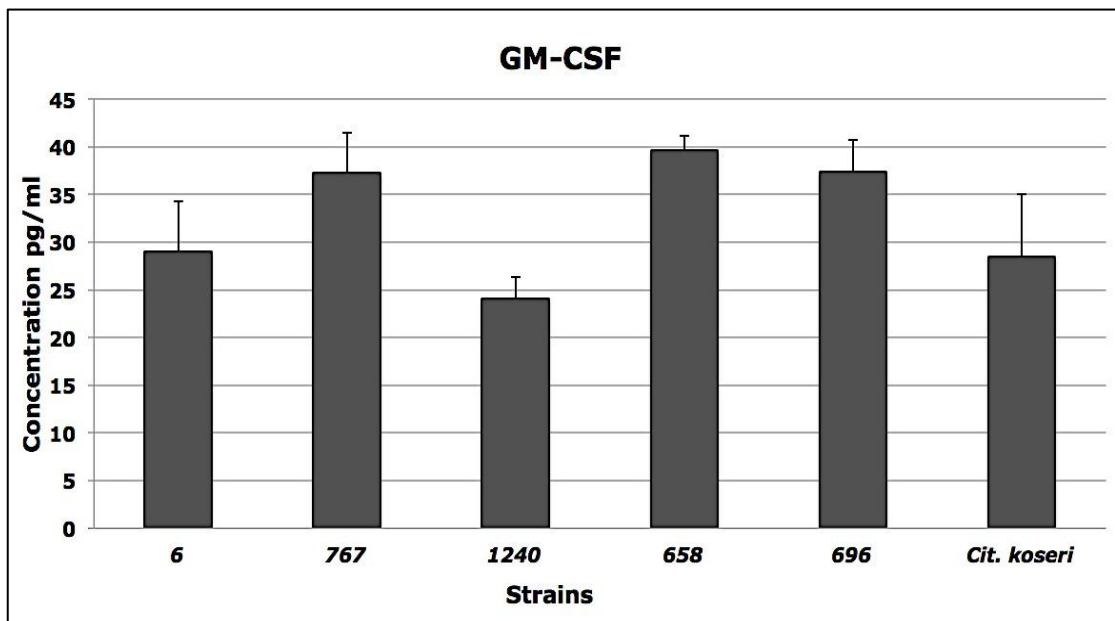

Fig.4.17. *C. sakazakii* induced GM-CSF expression in human cells. Human microglial cells (HMGC) were cultured in 24-well plates and infected with *C. sakazakii* strains for 5 hours. After collection of cell supernatants, GM-CSF levels were determined using Bio-Plex® ELISA. Values represent mean±SEM of triplicate determinations of two experiments.

A summary of the previous cytokine results in addition to invasion, translocation, and phagocytosis survival results using HBMEC and HMGC cell lines are provided in the tables below (Table.4.2-4).

Table.4.2: Summary of invasion, translocation, and phagocytosis survival results of *C. sakazkii* strains using HBMEC and HMGC cell lines.

Strain	ST	HBMEC invasion[1]	HBMEC translocation[2]	Microglia persistence[3]	Source	Clinical presentation	Risk of meningitis
20	4	High	High	P/M	Clinical	Unknown	High
695	4	High	High	P/M	Trachea	Fatal NEC II	High
721	4	High	Moderate	ND	CSF	Unknown	High
767	4	High	High	P/M	Trachea	Fatal meningitis	High
1219	4	High	High	ND	CSF	Fatal meningitis	High
1220	4	Moderate	High	ND	CSF	Brain abscess	High
1221	4	Moderate	High	P/M	CSF	Meningitis	High
1222	4	High	High	ND	Blood	Fever	High
1223	4	Moderate	High	ND	Blood	Unknown	High
1231	4	High	High	ND	Faeces	Meningitis	High
1240	4	High	High	P/M	CSF	Fatal meningitis	High
1587	109	High	ND	P/M	CSF	Brain damage	High
696	12	High	High	P/K	Faeces	NEC II	High
730	4	Moderate	Moderate	ND	Clinical	NEC I	Moderate
1224	4	Moderate	Moderate	ND	Blood	Fever	Moderate
1225	4	Moderate	Moderate	ND	Blood	Fatal meningitis	Moderate
1242	4	Moderate	Low	P/M	Brain	Fatal meningitis	Moderate
1249	31	Moderate	Low	P/M	Clinical	Fatal isolate	Moderate
1019	1	Moderate	Low	ND	CSF	Meningitis	Moderate
6	4	None	None	Killed	Clinical	Unknown	Low
553	4	Low	None	ND	Clinical	Unknown	Low
557	4	Low	Low	ND	Clinical	Unknown	Low
558	4	Low	None	P/K	Clinical	Unknown	Low
1465	4	Low	None	ND	PIF	Unknown	Low
4	15[a]	Moderate	None	ND	Clinical	Unknown	Low
12	1	Low	None	ND	Faeces	Unknown	Low
555	1	Low	Low	ND	Clinical	Unknown	Low
658	1	Moderate	Low	P/K	NIF	Unknown	Low
1241	1	Moderate	Low	ND	Blood	Unknown	Low
1	8	Low	None	ND	Throat	Unknown	Low
5	8	Moderate	None	P/K	Clinical	Unknown	Low
680	8	Moderate	Low	ND	CSF	Unknown	Low
520	12	Moderate	Low	ND	Clinical	Unknown	Low
580	18	Moderate	Low	ND	Clinical	Unknown	Low

ST: sequence type. [a]**:** part of clonal complex 4 (CC4).
ND: not done. **CSF:** cerebrospinal fluid **PIF:** powdered infant formula.
NIF: non-infant formula. **NEC:** necrotising enterocolitis.
P/M: persisted/multiplied. **P/K:** persisted/killed.
[1]**:** Fig.3.14 Chapter.3.
[2]**:** Fig.3.20 Chapter.3.
[3]**:** Fig.3.25 Chapter.3.

Table.4.3: Summary of the cytokine concentrations (pg/ml) recovered from *C. sakazakii*-induced HBMEC.

| Strains | ST | Pro-inflammatory | | | | Anti-inflammatory | | GM-CSF (TS <5 pg/ml) |
		IL-1β (TS <5 pg/ml)	TNF-α (TS <1 pg/ml)	IL-6 (TS <1 pg/ml)	IL-8 (TS <5 pg/ml)	IL-10 (TS <1 pg/ml)	IL-4 (TS <1 pg/ml)	
6	4	29.68±1.31	4.69±0.50	916.01±36.05	465.96±19.85	2.41±0.08	0	15.93±0.90
767	4	47.06±2.40	72.92±5.08	1310.63±67.16	3991.05±391.63	2.66±0.29	127.08±8.48	40.53±3.87
1240	4	49.04±5.10	23.77±2.60	1588.19±170.97	4024.41±905.76	3.16±0.29	139.97±13.57	39.71±3.08
658	1	52.99±4.24	15.02±4.42	1792.46±161.50	480.24±82.84	3.90±0.26	135.85±6.75	45.20±4.76
680	8	48.30±4.07	8.04±1.09	421.95±50.59	274.36±23.77	3.25±0.44	54.36±17.44	27.48±1.14
696	12	53.58±3.46	16.12±5.63	1485.72±77.11	3638.99±455.19	3.28±0.16	127.58±6.22	53.58±3.10
Cit. koseri	NA	62.63±7.97	22.23±7.17	1361.23±135.38	3310.44±531.46	3±0.25	84.64±27.34	48.30±4.07

Values represent mean±SEM of triplicate determinations of two experiments.
ST: Sequence type.
IL: Interleukin.
TNF: Tumour necrosis factor.
GM-CSF: Granulocyte-macrophage colony stimulating factor.
TS: Test sensitivity.
pg/ml: Picogram/millilitre.
NA: Not applicable.

Table.4.4: Summary of the cytokine concentrations (pg/ml) recovered from *C. sakazakii*-induced HMGC.

| Strains | ST | Pro-inflammatory | | | | Anti-inflammatory | | GM-CSF (TS <5 pg/ml) |
		IL-1β (TS <5 pg/ml)	TNF-α (TS <1 pg/ml)	IL-6 (TS <1 pg/ml)	IL-8 (TS <5 pg/ml)	IL-10 (TS <1 pg/ml)	IL-4 (TS <1 pg/ml)	
6	4	34.95±5.15	69.58±8.67	318.24±51.13	394.09±64.54	2.52±0.14	54.61±9.85	29.08±5.17
767	4	50.56±3.14	129.38±30.93	1357.96±125.38	3435.90±419.76	3.22±0.17	113.01±8.56	37.28±4.21
1240	4	31.10±4.66	65.59±1.57	1080.67±101.79	1945.71±311.41	3.28±0.38	104.88±3.95	24.12±2.27
658	1	34.73±3.14	65.15±11.52	1142.11±198.70	2699.74±331.53	3.01±0.25	112.73±4.31	39.69±1.45
696	12	37.43±3.51	91.70±18.44	1009.11±91.35	1711.70±240.06	2.72±0.26	92.53±5.02	37.43±3.25
Cit. koseri	NA	28.51±6.45	84.46±13.77	1022.54±163.62	2824.47±431.55	2.48±0.28	79.14±17.28	28.51±6.52

Values represent mean±SEM of triplicate determinations of two experiments.
ST: Sequence type.
IL: Interleukin.
TNF: Tumour necrosis factor.
GM-CSF: Granulocyte-macrophage colony stimulating factor.
TS: Test sensitivity.
pg/ml: Picogram/millilitre.
NA: Not applicable.

4.4. Discussion

4.4.1. Human total iNOS immunoassay

Nitric oxide (NO) is an inflammatory mediator that has a fundamental role in pathogenesis and can induce apoptosis and necrosis by altering the tight junctions of the cells. Inducible nitric oxide synthase increased significantly during the inflammation process leading to increased NO production (Chokshi *et al.* 2008). Thus, human total iNOS immunoassay was carried out to measure the amount of iNOS secreted by *C. sakazakii*-infected HBMEC cell line. Most of *C. sakazakii* strains, including ST4 strains, were able to stimulate the cells to produce high levels of iNOS. However, ST4 strains 6, 558, and 1465 in addition to strains 4 (ST15), 12 (ST1), 658 (ST1), 1 (ST8), and 5 (ST8) showed the least iNOS induction. The strains that caused high levels of iNOS production showed moderate to high translocation levels through HBMEC cell line especially ST4 strains, while the strains with decreased induction demonstrated low or no translocation e.g. strains 6 (ST4; no translocation) and 658 (ST1; low translocation). However there were some anomalies in the results, as ST4 strains 553 and 557 in addition to strains 1249 (ST31), 555 (ST1), 1019 (ST1; meningitis case), 1241 (ST1), 680 (ST8), and 520 (ST12) caused high iNOS induction, whereas the translocation levels were low suggesting that these strains did not utilise NO to invade and translocate through the HBMEC cell line (see Fig.4.1 and Table.4.1). The anomalies that were noticed in this assay might be attributable to the differences in the strains' behaviour in host attachment and invasion. Moreover, as *C. sakazakii* is a new emerging pathogen there is not a rich literature on the detail of the strategies this organism uses to alter host barriers and avoid immune responses which consequently limits interpretation of the data obtained.

Hunter *et al.* (2009) reported that *Enterobacter sakazakii* (*Cronobacter* species not determined) was able to induce NO production in the rat IEC-6 cell line *in vitro* and rat enterocytes *in vivo*, which triggered apoptosis in these cells. The study showed that the inhibition of NO production using siRNA resulted in suppressed *E. sakazakii*-induced apoptosis of IEC-6 cells indicating that NO is accountable for triggering cell death via apoptosis. Another study by Emami *et al.* (2012) suggested that mice enterocyte

apoptosis was attributed to intestinal iNOS expression that led to elevated NO production following *C. muytjensii* infection. Moreover, it was shown that infected Caco-2 and IEC-6 cell lines expressed significantly elevated levels of iNOS when infected with *C. muytjensii* human isolates. Consequently, the strains were able to disrupt the tight junctions of intestinal epithelial cells (Liu *et al.* 2012b).

The aforementioned studies provided information regarding the induction of iNOS expression using human, rat, and mice intestinal cells. Moreover, they focused mainly on the effect of iNOS in the onset of NEC (Hunter *et al.* 2008, Hunter *et al.* 2009, Emami *et al.* 2012). As *C. sakazakii* is known for its ability to cause neonatal meningitis, it is important to investigate the effect of this organism on HBMEC cells as a component of the BBB. Unlike the previous studies, this research is the first that reported the ability of clinical and non-clinical *C. sakazakii* strains to induce iNOS production in HBMEC that could potentially lead to excessive production of NO at the site of inflammation. This in turn could lead to the apoptosis or necrosis of the cell line leading to alteration of the tight junctions of these cells and subsequently cause the translocation of the organism. Hence, facilitating bacterial migration to the brain parenchyma. In addition to the MTT test, this experiment gave strong evidence for the ability of *C. sakazakii* strains to cause a cytotoxic effect and induce cell death *in vitro*, suggesting that they may be able to cross host barriers using these mechanisms *in vivo*.

4.4.2. Apoptosis marker detection

Previous studies have shown that *C. sakazakii* strains were able to induce apoptosis in different cell lines and infection models. These include human Caco-2 and rat IEC-6 cell lines in addition to mice and rats as infection models (Hunter *et al.* 2008, Emami *et al.* 2012, Liu *et al.* 2012b). Although these studies were able to demonstrate the ability of the organism to induce apoptosis, they nevertheless focused mainly on animal gut injury and NEC models. Moreover, Hunter *et al.* (2008) and Emami *et al.* (2011) used a miss-assigned strain (*C. muytjensii* strain 51239). The information regarding *C. sakazakii* apoptosis induction in human brain cell

lines is not available in the literature and this research is the first to discuss this behaviour.

All *C. sakazakii* strains that were used in this experiment exhibited the ability to induce apoptosis in HBMEC and HMGC cell lines, however strains 6 (ST4) and 658 (ST1) showed low fluorescence signals indicating low induction levels. Two different apoptotic markers were detected; caspase-3 and annexin V (Fig.4.2 and Fig.4.3). Caspase-3 is known as an important factor in apoptosis that is responsible for the proteolytic cleavage of many essential proteins including the nuclear enzyme poly ADP-ribose polymerase (PARP) (Cohen 1997). Moreover, activated caspase-3 is able to cleave and inactivate the inhibitor of caspase activated DNase (ICAD) allowing caspase activated DNase (CAD) to enter the nucleus and degrade chromosomal DNA (Gao and Kwaik 2000). Annexin V is an early apoptosis detection marker. It is a calcium-dependent phospholipid-binding protein that binds phosphatidylserine (PS) in the plasma membrane with high affinity. In early apoptosis, apoptotic cells lose their phospholipid membrane asymmetry and translocate PS rapidly to the outer leaflet of the plasma membrane where annexin V can bind (van Engeland *et al.* 1996, Zhang *et al.* 1997). The detection of this marker is an indication of the induction of apoptosis in the cell lines due to the changes in their plasma membrane.

The above mentioned results demonstrated that *C. sakazakii* is able to induce apoptosis in human brain cell lines (HBMEC and HMGC). This however did not explain the mechanism and the role of the host factors in this phenomenon. Gram-negative bacteria use different strategies by which they exploit host responses in order to cause damage. It was reported that *Chlamydia pneumoniae* was able to cause neuronal death via apoptosis through the induction of microglial TNF-α and IL-6 secretion, and by using neutralising antibody against TNF-α and IL-6, rat neuronal death was reduced by 50% (Boelen *et al.* 2007). Moreover, it was shown that the pathogenic strains of *Neisseria meningitidis* were able to induce TNF-α production in human epithelial cells, which activates the TNF receptor TNFR1 that induces apoptosis via triggering the caspase cascade

leading to the activation of caspase-3 induction (Gao and Kwaik 2000, Deghmane *et al.* 2009). In addition, the meningococcus induced cell death of HBMEC cells through NO and the signs of apoptosis including caspase-3 activation, DNA fragmentation, and phosphatidylserine translocation were observed in these cells (Schubert-Unkmeir *et al.* 2007). *Escherichia coli* K1 is another organism that is known for its ability to cause neonatal meningitis. It was reported recently that the Hcp1 protein that is secreted by a type VI secretion system was able to induce apoptosis in HBMEC cells via the activation of caspase-8 and subsequently caspase-3 in addition to causing IL-6 and IL-8 production (Zhou *et al.* 2012) Furthermore, *Klebsiella pneumoniae* was able to induce apoptosis in neurons through the up-regulation of IL-1β, IL-6, and TNF-α production by microglia, and the down regulation of these cytokines via melatonin administration decreased apoptosis and engendered neuroprotection (Wen *et al.* 2007, Wu *et al.* 2011, Parthasarathy and Philipp 2012).

Induction of apoptosis in HBMEC and HMGC cells suggest that this mechanism may be important for the organism to overcome the host barrier of the endothelium, and avoid the immune response in the brain associated with microglia. It was shown in this project that HBMEC and HMGC cell lines were able to produce TNF-α as a result of *C. sakazakii* infection (Table.4.3-4). Moreover, it was demonstrated that the organism was able to induce apoptosis and caspase-3 induction was detected (Fig.4.3). The proposed mechanism of *C. sakazakii*-induced apoptosis is that the TNF-α, which was produced by the endothelial and microglial cell lines binds TNFR, which in turn triggers the caspase cascade leading to the activation of caspase-3 that cleaves ICAD allowing CAD to degrade chromosomal DNA. Another potential mechanism, utilises the Fas ligand that is produced by activated killer cells of the adaptive immune system such as T cytotoxic lymphocyte to bind Fas, which is a member of the TNF receptor family, on the surface of the infected cell. This binding activates Fas leading to triggering of the caspase cascade allowing the activation of the executer caspase-3 that triggers cellular DNA degadation. Furthermore, the up-regulation of IL-1β, TNF-α, IL-6, and IL-8 expression induces acute inflammatory responses, such as the activation of immune

cells including macrophages, that might trigger apoptosis in HBMEC and HMGC cell lines *in vitro* and neurons *in vivo*. However, the aforementioned mechanisms are speculations that are not proven yet, and therefore future research is required to identify the exact mechanisms involved.

4.4.3. Cytokine production detection and profiling

Cytokine production by the infected host cells is important in order to eliminate bacterial infection. It works as an alarm system to recruit the immune system cells and components to fight this kind of invasion to protect the host. Many cytokines and chemokines can be secreted at the time of infection, depending on the site of the infection and the type of the invading organism. Moreover, each cytokine has its function in clearing the infectious agent and regulating the host responses. Any bias in these responses might contribute to an inability to eliminate the pathogen and might also increase the damage to the host tissues. HBMEC and HMGC cell lines were tested for cytokine production induction by *C. sakazakii* strains. The response of these cell lines towards the infection *in vitro* might help to understand the response of the host *in vivo* in case of central nervous system (CNS) infection that could lead to meningitis.

The experiment was designed initially to detect 10 human cytokines including GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α. Encouragingly, *C. sakazakii* strains were able to trigger the secretion of seven cytokines; IL-1β, IL-6, GM-CSF, TNF-α, IL-4, IL-8, and IL-10, which indicates that the organism triggered selective responses that can be advantageous to understand the host response during *C. sakazakii* CNS infection.

C. sakazakii strains demonstrated different levels of cytokine induction in HBMEC and HMGC cell lines. Regarding HBMEC cells, it was notable that there were no significant differences in cytokine induction with regard to the ST of the strains, as the strains from different STs showed similar behavaiour. Despite this similarity among all STs, ST4 strains 767 and 1240 showed highest levels of TNF-α induction, while strains 6 (ST4) and 680 (ST8) consistently induced low levels of most of the detected cytokines.

With regard to HMGC cells, although the significance in cytokine induction in this experiment was not linked to any specific ST, strain 767 (ST4) the strain that was responsible for a fatal meningitis case showed the most significant levels of induction for most of the detected cytokines. Moreover, the cytokines detected as a result of *C. sakazakii* infection were the same in both HBMEC and HMGC cell lines indicating that the organism triggered a selective host response, which in turn might cause severe consequences to the host.

IL-1β is one of the important pro-inflammatory cytokines that has a pivotal role in host defense against infectious agents and the pathogenesis of numerous inflammatory diseases. It is synthesised as an inactive form of cytoplasmic precursor that is proteolytically activated to the mature form in response to several pro-inflammatory stimuli such as caspase-1 (Franchi *et al.* 2006). Moreover, it is the only inflammatory cytokine that is closely related to TNF-α biologically (Dinarello 1993). It was reported that IL-1β and TNF-α were found mostly in bacterial meningitis not in aseptic or viral meningitis, which means that these cytokines are triggered by bacterial infection, and they are supposed to distinguish bacterial meningitis from viral (Leist *et al.* 1988, Nadal *et al.* 1989). Furthermore, they have a major role in the inflammation of the brain endothelium, and the inflammatory processes modify the metabolism and the dynamics of the cerebrospinal fluid (CSF), which leads to increased neurological damage (Ohga *et al.* 1994). According to Firestein *et al.* (2012), IL-1β is responsible for an increase in iNOS induction that could lead to increase NO production in tissues. This increase has a major impact on the cell line integrity and can result in cell line permeability by altering the tight junctions and initiating apoptosis.

Moreover, both IL-1β and TNF-α have roles in expression of endothelium adhesion molecules. They can up-regulate the expression of the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) that are not normally expressed on the surface of non-stimulated endothelial cells. These molecules facilitate the adhesion of the leukocytes at the endothelium of the inflamed tissue and allow the

migration of these cells from blood to the site of infection (Ockenhouse *et al.* 1992, Bevilacqua 1993, Jersmann *et al.* 2001). It was shown by Jersmann *et al.* (2001) that TNF and the lipopolysaccharide (LPS) of Gram-negative bacteria work synergistically to increase the adhesive properties of endothelial cells. Furthermore, these inflammatory mediators have the ability to induce ICAM-1 and VCAM-1 expression significantly.

A previous study showed that activated macrophages play a major role in IL-1β activation and acute inflammatory response in *Shigella* infection, and the apoptotic macrophages release active IL-1β that in turn triggers the inflammatory cascade and its cytokines such as TNF-α, IL-6, and IL-8 in addition to IL-1β (Zychlinsky and Sansonetti 1997).

It was shown previously in this research that the ability of *C. sakazakii* to induce apoptosis and caspase-3 activity was detected in HBMEC and HMGC cell lines that represent two barriers of the human brain. Moreover, it was demonstrated that some *C. sakazakii* strains were able to survive and avoid phagocytosis within human microglial cells, which are brain resident macrophages. Thus, it is proposed that *C. sakazakii* strains can activate HMGC cells inside the brain leading to the synthesis of IL-1β and TNF-α in addition to endothelial cells activation. Moreover, these strains are able to translocate through HBMEC cells, infect resident microglia, and avoid phagocytosis. This process could lead to apoptosis in these cells, as shown previously, and subsequently release the activated IL-1β that in turn triggers the acute inflammatory response. Additionally, inducing IL-1β production is another indication of apoptosis in these cells, as it needs apoptosis induction in order to activate caspase-1, and consequently activates IL-1β. Moreover, IL-1β and TNF-α expression by both endothelial cells and microglia enhances leukocyte adhesion to BBB endothelium, which is the step that comes before their migration to the CNS.

A study by Emami *et al.* (2012) examined the ability of a strain of *C. muytjensii* (strain ATCC 51239) to induce IL-1β and TNF-α production in mice fed with the bacterium using sera and intestinal homogenates. The results showed that the organism was able to stimulate the animal to produce these cytokines at high concentrations (>1000 pg/ml). However,

this study claimed that they used a *C. sakazakii* strain, and this might affect the validity of the results and the comparison. Moreover, those results were obtained from animal samples that had experimental NEC, while the results that were acquired from this research used human brain cell lines (endothelial cells and microglia). Townsend *et al.* (2007b) reported that *Cronobacter* was able to induce TNF-α expression and strain NTU84 (*C. dublinensis* ST43) induced high levels (500 pg/ml after 24 hours) from human macrophages. In this research, *C. sakazakii* strain 767 (ST4) was the most robust in TNF-α induction over 5 hours of incubation secreting >70 pg/ml with HBMEC and >120 pg/ml with HMGC. Although this project used two different cell lines than the one used in the previous publication, the results of the human macrophage cell line support the virulence potential of *Cronobacter* to stimulate the host to produce this pro-inflammatory cytokine that has a major adverse impact on the host infected tissues.

Cruz-Córdova *et al.* (2012) suggested that the flagella of *C. sakazakii* have the ability to trigger different immune responses in the host. The study used human macrophage cell line supernatants exposed to flagella and flagellin for 24 hours. TNF-α expression was detected, and the highest concentration was more than 400 pg/ml. This is another confirmation of the ability of the organism to induce severe host response that could in turn adversely affect the host tissues. Cytokines produced by macrophages will increase endothelium adhesion molecules expression that leads to the adhesion and migration of the leukocytes to the site of infection. This in turn might allow more bacterial cells to translocate with the white blood cells, allowing more bacterial load at the infected area. Moreover, the last study (Cruz-Córdova *et al.* 2012) reported that reducing the concentration of the flagella and flagellin led to a decrease in the concentration of different cytokines including IL-8, IL-10, and TNF-α, suggesting that the absence of flagella could lead to a weak host response. Their study did not include any non-motile strains, however in this research the non-motile strain 680 (ST8) was used to infect HBMEC cell line. The induction levels of TNF-α (<9 pg/ml; Fig.4.5) and IL-8 (<280 pg/ml; Fig.4.7) as a response to the exposure of this strain were >50%

lower when compared with the invasive motile strains, such as 767 (ST4), in this project. In addition, 680 did not exhibit significant induction of the anti-inflammatory cytokine IL-10 (<4 pg/ml; Fig.4.8). These findings suggest that flagella might play a critical role in host inflammatory response stimulation.

As stated in Ohga *et al.* (1994), the range of IL-1β levels were 80 – 5550 pg/ml in the CSF samples of 15 patients with bacterial meningitis. Moreover, the range of TNF-α levels of 20 patients suffered from bacterial meningitis were 0 – 8034 pg/ml (Dulkerian *et al.* 1995). The expression levels of IL-1β (Fig.4.4 and Fig.4.11) and TNF-α (Fig.4.5 and Fig.4.12) that were detected in this project *in vitro* are in concordance with the biological levels of these cytokines that were detected in cases of bacterial meningitis, suggesting that the *in vitro* data reflects the effects seen in *C. sakazakii*-induced meningitis.

IL-6 is a multifunctional cytokine that is locally produced in tissues as a response to different stimuli including bacterial infection. It has an essential role in immunity generation against intracellular infections (Kishimoto *et al.* 1992, Xing *et al.* 1998). IL-6 is essentially produced by endothelial cells, astrocytes, and monocytes in response to IL-1, and it can be present in CSF for longer periods than the other cytokines. IL-6 has predominantly pro-inflammatory effects, and it is a potent inducer of fever, acute-phase proteins, leukocytosis, clotting cascades, and complement activation (Hirano *et al.* 1990, Leib and Täuber 1999). A study by Hunter *et al.* (2008) demonstrated that *C. muytjensii* strain ATCC 51239 was able to induce IL-6 expression in IEC-6 cells and infected rat pups. The results showed IL-6 secretion in rat pups four days post infection (<150 pg/ml), while the highest concentration recovered from IEC-6 supernatants was after 12 hours of infection (<10 pg/ml). Moreover, the results obtained in this project of IL-6 production using HBMEC and HMGC cell lines were very high (>300 pg/ml; Fig.4.6 and Fig.4.13) compared with the results obtained by *C. muytjensii* induction.

Townsend *et al.* (2007b) showed that *Cronobacter* strains were able to stimulate human macrophages to secrete IL-6, and strain NTU84 (*C.*

dublinensis ST43) induced highest secretion >1100 pg/ml. As reported by Azuma *et al.* (1997), the range of IL-6 levels in CSF samples of 8 patients who suffered from bacterial meningitis were 209 to > 3500 pg/ml. This is in agreement with the data obtained in this research, which showed concentrations exceed 1700 pg/ml for HBMEC cell line and 1400 pg/ml for HMGC cell line (Fig.4.6 and Fig.4.13). These results suggest that *C. sakazakii* strains are capable of stimulating different host cells to induce acute inflammatory responses. This is potentially as a result of IL-6 induction that has a predominant pro-inflammatory effect and could cause leukocytosis at the site of the infection, as it is mainly produced locally in tissues. IL-6 might have destructive effects in cases of *C. sakazakii*-induced meningitis, as the organism was linked to severe meningitis cases including fatal infections (Caubilla-Barron *et al.* 2007, Joseph and Forsythe 2011). The induction of IL-6 expression might be attributed to IL-1β secretion of endothelial cells and microglia as a response to *C. sakazakii* infection suggesting that the infection is able to trigger a cascade of host responses that are involved in destructive effects.

IL-8 is an inflammatory mediator and a well-known member of a large family of chemokines that are broadly studied because of their primary activity to stimulate and attract leukocytes to the site of inflammation. It is produced by endothelial cells, macrophages, microglia, monocytes, and neurons as a response to bacterial products, IL-1β, and TNF-α stimulation (Baggiolini *et al.* 1993, Baggiolini *et al.* 1995, Sprenger *et al.* 1996). Moreover, IL-8 boosts neutrophil adhesion to endothelial cells as a prerequisite for leukocyte invasion into the brain and allows their migration (Leib and Täuber 1999, Täuber and Moser 1999). It was reported by Halstensen *et al.* (1993) that IL-8 was detected in the sera and CSF samples of 45% of tested patients suffered from meningococcal meningitis and bacteraemia. Furthermore, it was shown by the same study that IL-8 levels peaked after TNF and IL-1 induction and simultaneously with IL-6. Sansonetti *et al.* (1999) suggested that IL-8 is responsible for the trans-epithelial translocation of *Shigella* in rabbits besides its function in attraction of PMNs. Another study by Galanakis *et al.* (2006) reported that meningitis-causing *E. coli* K1 was able to up-

regulate IL-8 induction in HBMEC cells but not in human umbilical vein endothelial cells indicating that BBB endothelial cells show a unique response to meningitis-causing pathogens, as the non meningitis-causing *E. coli* strain HB101 did not show the same response.

There is only one publication that investigated the ability of *C. sakazakii* flagella of two different strains to trigger IL-8 in human macrophages cell line (Cruz-Córdova *et al.* 2012). It however did not investigate the host response using the live bacterial cells. It was shown previously in this research that *C. sakazakii* strains were able to induce IL-8 expression and it was the cytokine secreted in highest concentrations by HBMEC and HMGC cell lines. According to Sprenger *et al.* (1996), the average of the biological levels of IL-8 in the CSF samples of 14 patients with bacterial meningitis was 706 pg/ml, and the levels detected in this project exceed those levels (Fig.4.7 and Fig.4.14). Therefore, we speculate that IL-8 is produced as a response to the production of the pro-inflammatory cytokines IL-1β and TNF-α by endothelial cells and microglia. This induction of IL-8 aids the adhesion of leukocytes, and neutrophils in particular, to brain endothelium and allows their migration to the brain. During this process, bacterial cells in the blood stream could take advantage of the permeable endothelium and translocate to the CNS. IL-8, as a part of the host response, plays a critical role in the progression of *C. sakazakii*-induced meningitis by contributing to BBB permeability. This suggests that the severity of the brain infection caused by the organism might be attributable to the host response and not only to the virulence potential of the bacterium.

IL-10 is an anti-inflammatory cytokine that plays an important role in infection and inflammation. IL-10 is able to inhibit IL-8, IL-6, IL-1β, and TNF-α production *in vitro* and attenuates brain oedema during meningitis (París *et al.* 1997, Couper *et al.* 2008). Moreover, IL-10 decreases macrophage cytokine release, inhibits iNOS induction, and opposes the inflammatory effects of IL-1β and TNF-α, which can lead to reduced adhesion molecule expression, major histocompatibility complex (MHC), and matrix metalloproteinases (MMPs) release (Firestein *et al.* 2012).

Townsend *et al.* (2007b) showed the ability of *Cronobacter* strains to induce IL-10 production especially *C. sakazakii* strain 658 (ST1), which showed the highest secretion at 6 (>400 pg/ml) and 24 hours (>700 pg/ml) using human macrophages cell line. These findings, however do not correlate with the results obtained from this research, as strain 658 (ST1) in addition to the other strains failed to induce such high concentrations of IL-10 (<4 pg/ml) in HBMEC and HMGC cells over 5 hours of exposure when compared to the previous study (Fig.4.8 and Fig.4.15). This suggests that the anti-inflammatory effects of IL-10 in this project on *C. sakazakii* infection is weak, thus it could not attenuate the acute inflammatory responses caused by the pro-inflammatory cytokines including IL-1β, TNF-α, IL-6, and IL-8. This potentially keeps the inflammatory cascade active and able to induce more damaging responses.

IL-4 is one of the cytokines that has a dual role in immune response. It is a potent B lymphocytes activator and is involved in T helper 2 (Th2) lymphocyte and maturation. Moreover, it contributes in eosinophil migration and endothelial activation. On the other hand, it has an anti-inflammatory role that helps in decreasing Th2 cell and B cell apoptosis and suppressing IL-1β, and TNF-α production (Keelan *et al.* 2003, Dinarello 2007, Firestein *et al.* 2012). The lack of publications concerning the ability of *C. sakazakii* strains to induce IL-4 production did not help in explaining the role of this cytokine in infection. According to the results that were obtained from this research (Fig.4.9 and Fig.4.16), it is suggested that IL-4 did not affect the expression of IL-1β and TNF-α, which means that it was not capable of suppressing the pro-inflammatory process and it has weak anti-inflammatory response. Moreover, it might contribute in the activation of the BBB endothelium and facilitate the adhesion and the migration of leukocytes making the barrier permeable. Hence, the host response in the case of IL-4 contributes to the damage of the infected tissue rather than clearing the infection.

GM-CSF is a glycoprotein that has diverse effects on immune and non-immune cells. Moreover, it has the ability to stimulate T lymphocytes and

endothelial cells. It initiates the differentiation and proliferation of granulocytes and macrophages (Burgess and Metcalf 1980, Ruef and Coleman 1990). It is a cytokine that stimulates the antibacterial function of neutrophils and monocytes (Carr *et al.* 2003). It can be produced by endothelial cells as a response to IL-1 production to activate polymorphonuclear cells (PMNs) and allow their adhesion to endothelial surfaces (Hamilton 2002). Our research is the first to examine the ability of *C. sakazakii* to induce GM-CSF production. The concentrations of GM-CSF that were produced by HBMEC and HMGC cells were not high (<55 pg/ml; Fig.4.10 and Fig.4.17). It is proposed that this cytokine contributes in the activation and proliferation of macrophages and microglia at the time of *C. sakazakii* infection. Moreover, as a response of IL-1β production by endothelial cells, it might collaborate in the adhesion of PMNs to endothelial surfaces and facilitate their trans-endothelial migration that can be exploited by the organism to translocate to the CNS. Moreover, the activation of PMNs could cause a harmful effect on the host brains cells if they are recruited to eliminate the infection without a controlled inflammatory response.

In summary, *C. sakazakii* strains were able to induce pro-inflammatory responses in HBMEC and HMGC cell lines by stimulating the secretion of IL-1β, TNF-α, IL-6, and IL-8. Moreover, the anti-inflammatory cytokines including IL-10 were secreted at low concentrations and failed in their role to suppress the inflammatory process. It was notable that ST4 strains 767 and 1240 were the most significant inducers for the pro-inflammatory cytokines especially with HMGC cells. These strains were associated with two fatal meningitis cases (Table.4.2). However, strain 6 (ST4) did not follow the same pattern of induction as the previous strains suggesting its low virulence potency. This strain showed low invasion, translocation and phagocytosis survival levels (Table.4.2). Although strains 658 (ST1) and 696 (ST12) showed significant patterns of induction, those were not consistent through all cytokines and cell lines. They demonstrated low TNF-α and IL-8 induction with HBMEC, and low IL-1β and TNF-α induction with microglia, which was not noted with ST4 strains 767 and 1240. Additionally, strain 680 (ST8) the non-motile strain, induced low levels

cytokines supporting the importance of flagella in triggering the host response. Although strain 680 is a CSF isolate, it did not however show significantly elevated induction of the pro-inflammatory cytokines from HBMEC cells. Furthermore, strain 767 (ST4 CSF and fatal meningitis isolate) induced higher levels of pro-inflammatory cytokines than *Cit. koseri*, which is known for causing neonatal meningitis (Pollara *et al.* 2011). This suggests that *C. sakazakii* is able to induce some similar host responses as the meningitis-causing *Cit. koseri*, which might contribute to the pathogenesis of the disease. Generally, *C. sakazakii* ST4 meningitis strains 767 and 1240 were able to induce a greater pro-inflammatory response in the HBMEC and HMGC cells than the other strains from different STs.

Cytokine production is part of the inflammatory response at the site of infection. These molecules play an important role in controlling and clearing the infection. However, uncontrolled secretion by the mammalian cells could contribute to severe inflammatory response causing more damage to the organs and the infected tissues. According to the results obtained previously, the pro-inflammatory response out weigh the anti-inflammatory one, as the anti-inflammatory cytokines including IL-4 and IL-10 did not inhibit the pro-inflammatory ones such as IL-1β, IL-6, IL-8, and TNF-α. In addition, granulocyte-macrophage colony stimulating factor (GM-CSF) was induced by *C. sakazakii* strains. This cytokine could be involved in the activation of macrophages and microglia, which might lead to elevated induction of the inflammatory cytokines. The pro-inflammatory cytokines were detected in patients with bacterial meningitis indicating that they have a major impact on the CNS. They are involved in the adhesion of the leukocytes especially neutrophils to the endothelial surfaces and allow their migration to brain parenchyma by causing endothelium permeability. Moreover, they activate and attract the immune cells to eliminate the source of the infection. All these processes are intended to protect the host and eradicate the infectious agent. Nevertheless, they might help the bacteria to gain access through the BBB and reach the brain to cause massive damage. Furthermore, they induce other responses that include fever and CSF biological changes that could

lead to neuronal damage. This strategy of the host defense ends by harming the host instead of fighting the infection and making the host response an important factor that contributes in host damage in addition to the bacterial traits.

Chapter 5: General discussion

5.1. Justifications and summary of work

Thirty-four *C. sakazakii* strains from different sources have been analysed many of which were previously studied primarily with respect to phylogeny and to a lesser extent pathogenicity (Caubilla-Barron *et al.* 2007, Townsend *et al.* 2007b, Giri *et al.* 2011, Joseph and Forsythe 2012c). These strains represent different sequence types and were taken from clinical samples such as CSF and blood in addition to non-clinical samples such as PIF (Table.2.3-4). Most of the clinically important strains are within clonal complex 4 (CC4; Joseph and Forsythe 2012c). The experiments were performed on all CC4 and non-CC4 strains using consistent methodologies to permit rational comparisons. .

Townsend *et al.* (2007b) examined the pathogenicity and virulence of *C. sakazakii* strains, but that study did not include the HBMEC line to assess the ability of the organism to penetrate the BBB. These virulence studies were on a group of strains belonging to the French outbreak in 1994 and did not use strains from any other cases of severe infection. . In addition, although Giri *et al.* (2011) showed in their research the ability of *C. sakazakii* to invade and translocate through Caco-2 and HBMEC lines, they did not propose any mechanism that might affect the integrity of the polarised monolayers of these cell lines. Additionally, the vast majority of the strains used in that study were from environmental and food sources and the clinical isolates did not show significant virulence potential. Another study (Liu *et al.* 2012b) examined the induction of monolayer permeability and apoptosis of intestinal epithelial cells *in vitro*, but this research used mostly environmental or food isolates. They also included three improperly assigned *C. malonaticus* strains and one *C. dublinensis* strain as *C. sakazakii* strains; both issues might affect the validity of their findings and compromise the reliability of their study, at least to some extent. The clonal complexity of *Cronobacter* spp. was investigated in several studies using MLST (Joseph and Forsythe 2011, Joseph *et al.* 2012b, Joseph and Forsythe 2012c), and it was found that most of the sequence types (STs) belong to *C. sakazakii*, with the majority of the

clinical strains belonging to CC4. However, there were no follow-up studies that examined the differences in pathogenicity and virulence among *C. sakazakii* STs. Host response to *C. sakazakii* infection, including iNOS and cytokine production, and apoptosis induction, has been investigated previously (Hunter *et al.* 2009, Emami *et al.* 2012), but the organism was not *C. sakazakii* as reported; it was in fact *C. muytjensii* (strain 51239). Another study (Cruz-Córdova *et al.* 2012) tested the ability of *C. sakazakii* strains to induce human macrophage cells (U937) to produce cytokines, using flagella and flagellin for their experiments. The discussion above shows that the literature does not currently give a clear picture about the pathogenicity and virulence of *C. sakazakii*. Additionally, aspects regarding host response to infection demand further investigation. Moreover, most of the studies of host response to *C. sakazakii* infection did not use the correct organism and strains, despite what appears in the published literature. They also did not include human brain cells such as HBMEC and microglial lines to investigate the impact of, and response to infection.

Therefore, the work described in Chapter 3 aimed to examine the virulence potential of *C. sakazakii* strains using a range of tissue culture and non-tissue culture assays including molecular techniques, which enabled the detailed evaluation of the potential ability of the organism to establish a successful infection and overcome the host's intestinal barrier and blood brain barrier (BBB). The results of those virulence studies revealed a group of virulence traits that might increase pathogenicity. Although some strains were non-motile, the presence of functional flagella in motile strains might allow the bacterium to attach to abiotic surfaces and aid the adhesion to Caco-2 and HBMEC cells in addition to biofilm formation (Fig.3.1 and Table 3.1). The iron acquisition system encoded in the genome of the sequenced strains, along with the ability of the organism to produce iron siderophores, are important to maintain its growth *in vitro* and might support the growth *in vivo* (Fig.3.2 and Table 3.2). Despite the fact that some strains were sensitive to human serum and rapidly killed by phagocytes *in vitro*, some other strains were serum resistant and able to survive and multiply within phagocytes. Withstanding

serum-mediated killing and resisting phagocytosis might promote the survival of some strains in the bloodstream and might be advantageous for causing bacteraemia (Fig.3.3, 23, and 25 and Table 3.2-5).

The cytotoxicity of *C. sakazakii* strains can affect the integrity of cell monolayers, as the strains showing higher cytotoxicity for Caco-2 and HBMEC lines were found to be highly translocated through these cell lines and led to a decreased transepithelial resistance (TEER), an indication of tight junction disruption (Table.3.5, Fig.3.18, and Fig.21). Additionally, *C. sakazakii* cytotoxicity might have a major impact on the immune response when the organism crosses the BBB, as it was shown that the high cytotoxicity led to a decrease in the number of viable microglial cells (Table.3.5 and Fig.3.7). The organism demonstrated the ability to attach to Caco-2, HBMEC, and rBCEC4 lines, and the strains of CC4 (Caco-2 2.55%, HBMEC 2.42%, and rBCEC4 2.9%) showed higher attachment than non-CC4 strains (Caco-2 2.05%, HBMEC 1.59%, and rBCEC4 2.6%), which could be of assistance for some strains in invading these cell lines (Fig.8, 9, and 12). The invasive ability of *C. sakazakii* strains was also demonstrated in this study, and CC4 strains (Caco-2 0.29%, HBMEC 0.13%, and rBCEC4 0.02%) were more invasive in comparison with non-CC4 strains (Caco-2 0.16%, HBMEC 0.1%, and rBCEC4 0.015%; Fig.12, 14, and 16). The bacterium also demonstrated the ability to translocate through the Caco-2 and HBMEC monolayers. There were no differences in the translocation ability between CC4 and non-CC4 strains with regard to the Caco-2 cell line, while most of the strains that translocated at high levels through HBMEC cells were in the CC4 group (HBMEC 4.92%, non-CC4 1.67%; Fig.3.17 and Fig.3.20). Although most of the invasive and highly translocated strains belong to CC4, there were some CC4 strains that only invaded and translocated to a lower degree (Table.3.4). This might be attributed to genetic variation in the strains in this clonal complex, but it is also possible that these strains lost their virulence traits as a result of the subculture process or conditions in the laboratory. The translocation of the organism through intact polarised monolayers of Caco-2 and HBMEC *in vitro* might be an indication of the ability of *C. sakazakii* to overcome host physical barriers in the gut and brain *in vivo*.

This translocation might also play a major role in the pathogenesis of NEC and meningitis by inducing inflammatory mediators that might contribute to tissue damage.

In addition, *C. sakazakii* strains were able to survive within human macrophages and microglial cells (Fig.3.23 and Fig.3.25). Although some strains survived the first impact with these cells, they were killed rapidly following uptake. Despite that, other strains were able to survive and multiply within these phagocytes, and some of these strains were linked to severe and fatal neonatal infections (Himelright *et al.* 2002, Caubilla-Barron *et al.* 2007). The ability to withstand phagocytosis provides a shelter within phagocytes for *C. sakazakii* strains by which they might evade immune responses and that could support their virulence and pathogenicity. The earlier summary suggested that *C. sakazakii* strains have a group of virulence traits and pathogenic mechanisms whereby they could invade the host and avoid protective barriers within the body. They also might influence the pathogenesis of the diseases that could be caused by the organism including NEC and meningitis.

The research that was described in Chapter 4 aimed to assess the host response to *C. sakazakii* infection using human brain cell lines. *C. sakazakii* strains were able to induce HBMEC cells to produce iNOS. Although there were some anomalies, the strains that produced high iNOS levels were highly translocated strains through HBMEC cell line (Fig.4.1). This highlights the role of iNOS production that could lead to high NO levels that might contribute to cell line permeability (Henry and Lawrence Moss 2010, Iben and Rodriguez 2011). The organism was also able to induce apoptosis in HBMEC and microglial cells. Two apoptotic markers were found, caspase-3 and annexin V (Fig.4.3). Inducing apoptosis in the BBB endothelium might allow further bacterial migration as the barrier loses its integrity. Induction of apoptosis in microglial cells might also reduce their ability to clear or attenuate the infection. A group of cytokines were produced by HBMEC and microglial cell lines as a result of *C. sakazakii* infection. Although 10 cytokines were tested, only 7 of these cytokines were detected. These include IL-1β, IL-6, GM-CSF, TNF-α, IL-4,

IL-8, and IL-10 (Table.4.3-4). The importance of these cytokines and their functions were discussed previously in the same chapter. These cytokines, as a part of the host response, might contribute to the host damage by mediating BBB permeability, leukocyte migration into the brain, the inflammation of the brain endothelium, and neurological injury. It was notable that the anti-inflammatory function of IL-10 failed to regulate the high concentrations of the inflammatory cytokines such as IL-6 and IL-8 *in vitro*. This suggests that the inflammatory process out weigh the anti-inflammatory one, and this could cause deleterious outcomes. However, *in vivo* more types of cells might be involved in the inflammatory response and further examination of the potential interactions of different cell types and the cytokines they generate is required to allow more accurate evaluation. The preceding summary proposed that *C. sakazakii* infection was able to stimulate the host to produce inflammatory mediators, which might contribute to the pathogenesis of meningitis. This could be via the increased levels of the inflammatory mediators and apoptosis induction. It also suggests that the pathogenesis of meningitis might be attributed to uncontrolled host response not the organism *per se*.

These data suggest that the ability of *C. sakazakii* to cause serious infection is likely to involve several bacterial virulence factors acting at different stages of the disease process. The strains analysed in this thesis show variability in traits which may contribute to disease and the data suggest that in some cases, different strains may use alternative mechanisms to achieve the same result, for example translocation across cell monolayers. Identification of the specific factors that account for the apparent increased virulence of strains in CC4 still requires further study. However, based on the data obtained to date, Fig.5.1 summarises the variability in strain behaviour observed in assays modelling the proposed sequential steps of *C. sakazakii*-host interactions during the pathogenesis of *C. sakazakii*-induced meningitis.
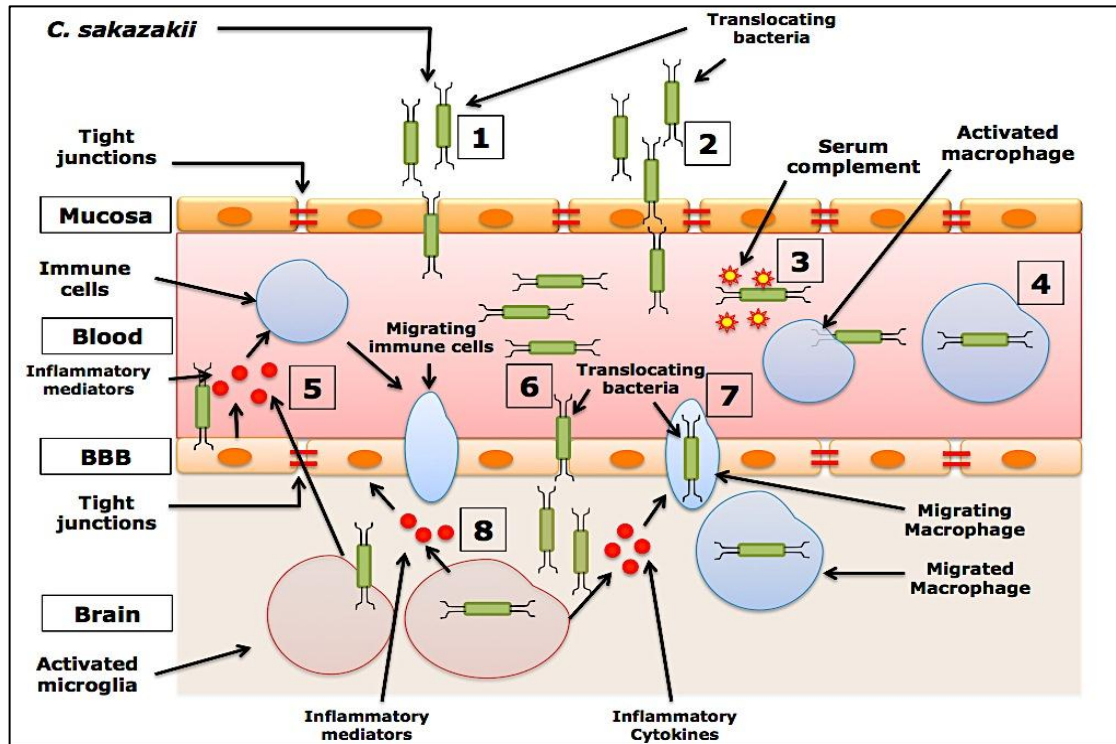
Fig.5.1. The proposed sequential steps of *C. sakazakii*-host interactions during the pathogenesis of *C. sakazakii*-induced meningitis and variable behaviour of *C. sakazakii* strains. **(1)** Paracellular translocation of bacterial cells through the disrupted tight junctions (of Caco-2 cells) that might have been affected by the cytotoxicity induced by *C. sakazakii* (strains 695, 721, 767, 1220, 1222, 1223, 1224, 1225, 4, 1249, 12, 658, 1241, and 696), the translocation of these strains was accompanied by a notable decrease in TEER. **(2)** Transcellular translocation of bacterial cells (strains 20, 553, 557, 558 730, 1219, 1221, 1231, 1240, 1242, 1465, 680, and 580) by invasion of gut cells (Caco-2) and translocation through them without a notable change in TEER. OmpA might play an important role in this step. **(3)** *C. sakazakii* (strains 20, 558, 695, 767, 1221, 1240, 1242, 1587, 1249, 658, and 696) survival in the bloodstream by avoiding complement-dependent killing of serum - possibly mediated through cleaving complement components C3 and C4b, by the *cpa* gene product. Moreover, some other factors such as iron uptake could support the survival of *C. sakazakii* in the host environment. **(4)** Intracellular survival and multiplication of *C. sakazakii* (strains 20, 695, 767, 1221, 1240, 1242, 1587, 1249, 658, and 696) in blood macrophages that help the bacterium to evade the immune response and work as a vehicle to transport the intracellular bacteria to other body sites. Gene products such as SodA might have an important role in this process. **(5)** *C. sakazakii* attachment and invasion (strains 20, 695, 721, 767, 1219, 1220, 1221, 1222, 1223, 1224, 1225, 1231, 1240, 1242, 1249, 1241, and 696) of brain endothelium (HBMEC cells) might lead to the release of some inflammatory mediators such as IL-1β, TNF-α, IL.6, IL-8, GM-CSF, and iNOS (that could lead to increased NO production). These might contribute to cell line permeability and the migration of several immune cells such as neutrophils and macrophages (pleocytosis). The cell line permeability might facilitate the translocation of *C. sakazakii* into the brain. **(6)** Two ways of *C. sakazakii* translocation into the brain are suggested; paracellular translocation (strains 20, 695, 721, 730, 767, 1219, 1220, 1221, 1222, 1223, 1224, 1225, 1231, 1240, 1242, 1249, 1241, and 696) by altering the tight junctions through cytotoxicity and apoptosis (caspase-3) induction, and by **(7)** using Trojan horse mechanism inside macrophages. **(8)** The translocated *C. sakazakii* strains could be phagocytised by microglia (HMGC cells) inside the brain and then survive and multiply (strains 20, 695, 767, 1221, 1240, 1242, 1587, and 1249). The activated microglia produce inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8 and GM-CSF, which might contribute to brain endothelium (HBMEC cells) permeability and by attracting more immune cells to migrate to the brain. The inflammatory process inside the brain might lead to neuronal death, increased intracranial pressure, and severe inflammation of the meninges. Strains in red belong to CC4 and strains in blue belong to non-CC4.

5.2. Limitations and future work

As discussed, *C. sakazakii* strains showed the ability to translocate through Caco-2 and HBMEC cell lines. Although the mechanism of the translocation was not clear, it was suggested in this project that the mechanism might include altering the tight junctions. Using electron microscopy to visualise the translocation process could provide further understanding of this process. Moreover, due to the lack of an experimental model that resembles human barriers, using a multilayer translocation assay, by which different human cells can be grown as layers to investigate the ability of the organism to pass through different host barriers, is required. Further optimisation for the translocation assays is recommended to eliminate any conditions that might affect results such as increasing the infection period, avoiding contamination, maintaining cell line integrity, and obviating the acidification of the infection medium. However, good translocation results were obtained from this project that showed a correlation between the invasiveness of the strains and the translocation ability.

The survival and multiplication of *C. sakazakii* strains within phagocytes was shown in Chapter 3, and this probably contributes to the pathogenicity of the organism. The strains showed persistence within macrophages up to 72 hours following their uptake. Despite some strains showing reduced numbers with time, the other strains were able to multiply and showed increased numbers. Other immune cells including polymorphonuclear leukocytes (PMNs) could be used to examine the survival of *C. sakazakii* strains. Also, increasing the frequency of sampling during assays might provide further knowledge about the survival rates of these strains. The working time regulations and safety considerations at the laboratory prevented the possibility of adding more testing time points to the experiment, and alternative experimental design might help to overcome this issue. The survival within microglia by test strains was demonstrated in the same chapter. Although some strains were rapidly killed, some strains were able to persist and replicate. The massive reduction in viability of some strains following the uptake by microglia needs further investigation to clarify the mechanism by which the

organism was eradicated (Fig.3.25 and Table.3.4). However, it was suggested in this research that this reduction might be attributed to the high cytotoxicity induced by the organism in these cells, which might led to their death and the release of the intracellular bacteria into the medium, which were then lost in the washing step of the assay. Moreover, it is important to investigate the genes responsible for the phagocytosis survival, and studying the ability of *C. sakazakii* strains to form a spacious phagosome that was previously found to mediate phagocytosis survival in *Y. pestis* is required (Oyston *et al.* 2000, Kukkonen *et al.* 2001, Grabenstein *et al.* 2006, Pujol *et al.* 2009). Nonetheless, this research suggested a group of genes that might promote phagocytosis survival (Table.3.2).

C. sakazakii strains were able to induce iNOS production in the HBMEC cell line, and this induction might lead to NO synthesis that could contribute to cell line permeability as discussed previously in Chapter 4. Investigating the amount of NO production at the time of infection and linking it to the levels of iNOS induction is needed to elucidate its role in the increase of NO production during infection. Although there were some anomalies, it was displayed in this project that the strains that induced high iNOS levels showed moderate and high translocation capacity through the HBMEC cell line. Inducing apoptosis in HBMEC cells affects the integrity of this line and allow further migration of bacterial cells. Investigating the apoptosis pathways that can be induced by the organism including additional apoptotic markers, such as poly-ADP ribose polymerase (PARP) and p53, is required to give a clear picture about the mechanism by which the organism could induce apoptosis. In addition further study of the ability of the organism to cause necrotic cell death that has an adverse effect on host tissues is needed. It was shown previously in Chapter 4, that *C. sakazakii* strains induced apoptosis in HBMEC and HMGC cell lines, and two apoptotic markers were detected including caspase-3. It was proposed that the induction of caspase-3 might result from the activation of TNFR by TNF-α, which leads to trigger the caspases pathway leading to caspase-3 induction (Gao and Kwaik 2000, Deghmane *et al.* 2009). Evaluation of the inflammatory process during the infection is important in

order to investigate the role of the host response in this process and its contribution in tissue damage. In Chapter 4, as formerly described it was shown that *C. sakazakii* infection triggered the production of 7 cytokines. The role of these cytokines in pathogenesis needs further investigation. Using antibodies to block the activity of these cytokines could be beneficial if applied in the translocation assay to determine their effect on cell line permeability. Moreover, examining the function of the anti-inflammatory cytokines in controlling the inflammatory process in addition to the role of the pro-inflammatory ones in attracting leukocytes is required. However, the preceding suggestions in this research proposed that these cytokines have a role in pathogenesis. The high levels of IL-8 induction in HBMEC and microglial cell lines resulted from the infection of the invasive and highly translocated strains indicating its impact on the cell line integrity. Brain endothelial cells are positioned and specially modified to perform the functions of BBB. These functions are aided by the polarisation of BBB properties such as protein receptors, lipid receptors, and lipid transporters. The latter are distributed unevenly and uniquely between luminal and abluminal membranes of brain endothelial cells. Thus, these cells have the potential to receive signals from one side e.g. blood and secrete to another e.g. brain and *vice versa*. This behaviour could mimic the *in vivo* conditions of the BBB including the presence of the tight junctions and the polarised transporters (Betz and Goldstein 1978, Deli *et al.* 2005, Verma *et al.* 2006). Therefore, it is important to study the response of polarised HBMEC cells to *C. sakazakii* infection that could illustrate the role of these polarised cells in infection and pathogenesis. Moreover, lipopolysaccharide (LPS) could be a potent inducer for cytokine production and might affect the permeability of the HBMEC cell line (Xaio *et al.* 2001, Verma *et al.* 2006). Hence, examining *C. sakazakii* LPS and its effects on HBMEC cells could clarify its role in the permeability of the BBB.

Due to the sensitivity of the cell lines used in this project and the high cost of analysis, which had affected the total budget towards the end of the project, limited options of assessment tests were used. Therefore, a possible improvements in future work could be the use of further

assessment assays incorporating appropriate negative and positive controls for the host response. These assays might include apoptotic markers detection and quantification via flowcytometry and immunoassays. They might also involve animal models to investigate *C. sakazakii* infection and host response *in vivo*.

5.3. Final summary and conclusions

This study has made clear that *C. sakazakii* has several virulence traits that allow it to establish a successful infection and evade host immune response. Moreover, the organism showed the ability to attach and invade a set of mammalian cell lines and induce cytotoxicity among them. Additionally, it was demonstrated that the organism was able to translocate through two human cell lines—Caco-2 and HBMEC—representing the gut and brain barriers respectively. Furthermore, the capacity of the bacterium to survive within macrophages and microglia, which indicates its ability to avoid phagocytosis, was observed. The organism was able to induce inflammatory mediators and apoptosis. It was proposed that host response could be an influential factor that might cause major damage to the tissues and support pathogenesis. It was also shown that CC4 strains have higher invasion and translocation abilities to HBMEC cells *in vitro* (HBMEC invasion 0.13%, HBMEC translocation 4.92%) compared to non-CC4 strains (HBMEC invasion 0.1%, HBMEC translocation 1.67%). This supports the previous conclusion that *C. sakazakii* CC4 was the most prevalent clonal complex of meningitis and CSF isolates (Joseph and Forsythe 2012c, Forsythe *et al.* 2014).

In summary, this study has provided evidence from a range of virulence and pathogenicity assessments that *C. sakazakii* is a virulent pathogen that can overcome host barriers, evade immune response, and survive phagocytosis. Hence, it can be a risk factor for neonates and immunocompromised patients. Moreover, host response assessments have highlighted the uncontrolled response that can be responsible for deleterious outcomes resulting from the infection. This highlights the need of effective therapeutic strategies that could control the host response during the infection and subsequently controlling the progressive damage

of the CNS caused by *C. sakazakii* infection. It was shown previously that dexamethasone could be used as an adjunctive therapy with antibiotics to reduce the concentration of IL-1β and TNF-α and consequently reduce the degree of meningeal inflammation. This also was found to improve the long-term outcome in infants and children with meningitis (Odio *et al.* 1991, Tunkel *et al.* 2004). It was also reported that using immunoglobulins and inhibitors of inflammatory cytokines might help to minimise neuronal damage and improve the host immune response. Moreover, the inactivation of caspase-3 using brain-derived neurotrophic factor might result in reducing neuronal apoptosis (van de Beek *et al.* 2006, Prasad *et al.* 2012). Therefore, designing a diagnostic and therapeutic plan for *C. sakazakii* infection could help in eliminating the consequences of the infection and reducing the damage resulted from the host response. Importantly, the outcomes of this project have also proposed possible areas of future research to follow that may broaden knowledge of the pathogenicity of the organism, host response consequences, and therapeutic strategies.

References

Adegbola, R. & Old, D. (1983). Fimbrial haemagglutinins in *Enterobacter* species. *Journal of General Microbiology,* **129,** 2175-2180.

Aguirre Conde, A., Pérez Legorburu, A., Echániz Urcelay, I., Hernando Zárate, Z. & Arrate Zugazabeitia, J. (2007). Sepsis neonatal por *Enterobacter sakazakii*. Anales de Pediatría. Elsevier Doyma, 196-197.

Aktan, F. (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sciences,* **75,** 639-653.

Allen, P., Hart, C. & Saunders, J. (1987). Isolation from *Klebsiella* and characterization of two rcs genes that activate colanic acid capsular biosynthesis in *Escherichia coli*. *Journal of General Microbiology,* **133,** 331-340.

Almuneef, M. A., Baltimore, R. S., Farrel, P. A., Reagan-Cirincione, P. & Dembry, L. M. (2001). Molecular typing demonstrating transmission of gram-negative rods in a neonatal intensive care unit in the absence of a recognized epidemic. *Clinical Infectious Diseases,* **32,** 220-227.

Amalaradjou, M. A. R. & Venkitanarayanan, K. (2011). Proteomic analysis of the mode of antibacterial action of trans-cinnamaldehyde against *Cronobacter sakazakii* 415. *Foodborne Pathogens and Disease,* **8,** 1095-1102.

Azuma, H., Tsuda, N., Sasaki, K. & Okuno, A. (1997). Clinical significance of cytokine measurement for detection of meningitis. *The Journal of Pediatrics,* **131,** 463-465.

Badger, J. L., Stins, M. F. & Kim, K. S. (1999). *Citrobacter freundii* invades and replicates in human brain microvascular endothelial cells. *Infection and Immunity,* **67,** 4208-4215.

Badger, J. L., Wass, C. A. & Kim, K. S. (2000). Identification of *Escherichia coli* K1 genes contributing to human brain microvascular endothelial cell invasion by differential fluorescence induction. *Molecular Microbiology,* **36,** 174-182.

Baggiolini, M., Dewald, B. & Moser, B. (1993). Interleukin-8 and related chemotactic cytokines—CXC and CC chemokines. *Advances in Immunology,* **55,** 97-179.

Baggiolini, M., Loetscher, P. & Moser, B. (1995). Interleukin-8 and the chemokine family. *International Journal of Immunopharmacology,* **17,** 103-108.

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C. & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology,* **9,** 223-231.

Ballabh, P., Braun, A. & Nedergaard, M. (2004). The blood–brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiology of Disease,* **16,** 1-13.

Bar- Oz, B., Preminger, A., Peleg, O., Block, C. & Arad, I. (2001). *Enterobacter sakazakii* infection in the newborn. *Acta Paediatrica,* **90,** 356-358.

Barichello, T., Fagundes, G. D., Generoso, J. S., Elias, S. G., Simões, L. R. & Teixeira, A. L. (2013). Pathophysiology of neonatal acute bacterial meningitis. *Journal of Medical Microbiology,* **62,** 1781-1789.

Barreira, E. R., Costa de Souza, D., de Freitas Góis, P. & Fernandes, J. C. (2003). Meningite por *Enterobacter sakazakii* em recém-nascido: relato de caso. *Pediatria (São Paulo),* **25,** 65-70.

Barron, K. D. (1995). The microglial cell. A historical review. *Journal of The Neurological Sciences,* **134,** 57-68.

References

Baumbach, J., Rooney, K., Smelser, C., Torres, P., Bowen, A. & Nichols, M. (2009). *Cronobacter* species isolation in two infants - New Mexico, 2008. *Morbidity and Mortality Weekly Report*. CDC. **58**, 1179-1183. Available: http://www.cdc.gov/mmwR/preview/mmwrhtml/mm5842a3.htm [Accessed 07/10/2014].

Baumgartner, A., Grand, M., Liniger, M. & Iversen, C. (2009). Detection and frequency of *Cronobacter* spp.(*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula. *International Journal of Food Microbiology,* **136,** 189-192.

Beaman, L. & Beaman, B. L. (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. *Annual Reviews in Microbiology,* **38,** 27-48.

Bell, M. J., Ternberg, J. L., Feigin, R. D., Keating, J. P., Marshall, R., Barton, L. & Brotherton, T. (1978). Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Annals of Surgery,* **187,** 1-7.

Bessman, M. J., Walsh, J. D., Dunn, C. A., Swaminathan, J., Weldon, J. E. & Shen, J. (2001). The Gene ygdP, Associated with the Invasiveness of *Escherichia coli* K1, Designates a Nudix Hydrolase, Orf176, Active on Adenosine (5')-Pentaphospho-(5')-adenosine (Ap5A). *Journal of Biological Chemistry,* **276,** 37834-37838.

Betz, A. L. & Goldstein, G. W. (1978). Polarity of the blood-brain barrier: neutral amino acid transport into isolated brain capillaries. *Science,* **202,** 225-227.

Bevilacqua, M. P. (1993). Endothelial-leukocyte adhesion molecules. *Annual Review of Immunology,* **11,** 767-804.

Bewley, M. A., Pham, T. K., Marriott, H. M., Noirel, J., Chu, H.-P., Ow, S. Y., Ryazanov, A. G., Read, R. C., Whyte, M. K. & Chain, B. (2011). Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*. *Molecular & Cellular Proteomics,* **10,** M111. 008193.

Biering, G., Karlsson, S., Clark, N. C., Jónsdóttir, K. E., Ludvigsson, P. & Steingrimsson, O. (1989). Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. *Journal of Clinical Microbiology,* **27,** 2054-2056.

Bishop, G. M., Dang, T. N., Dringen, R. & Robinson, S. R. (2011). Accumulation of non-transferrin-bound iron by neurons, astrocytes, and microglia. *Neurotoxicity Research,* **19,** 443-451.

Blanc- Potard, A. B. & Groisman, E. A. (1997). The *Salmonella* selC locus contains a pathogenicity island mediating intramacrophage survival. *The EMBO Journal,* **16,** 5376-5385.

Block, C., Peleg, O., Minster, N., Bar-Oz, B., Simhon, A., Arad, I. & Shapiro, M. (2002). Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. *European Journal of Clinical Microbiology and Infectious Diseases,* **21,** 613-616.

Boelen, E., Steinbusch, H. W., van der Ven, A. J., Grauls, G., Bruggeman, C. A. & Stassen, F. R. (2007). *Chlamydia pneumoniae* infection of brain cells: An *in vitro* study. *Neurobiology of Aging,* **28,** 524-532.

Bogard, R. W. & Oliver, J. D. (2007). Role of iron in human serum resistance of the clinical and environmental *Vibrio vulnificus* genotypes. *Applied and Environmental Microbiology,* **73,** 7501-7505.

Bowen, A. B. & Braden, C. R. (2006). Invasive *Enterobacter sakazakii* disease in infants. *Emerging Infectious Diseases,* **12**.

Brady, C., Cleenwerck, I., Venter, S., Coutinho, T. & De Vos, P. (2013). Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): Proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis*comb. nov.

and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia*gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Systematic and applied microbiology,* **36,** 309-319.

Braun, J., Novak, R., Herzog, K.-H., Bodner, S., Cleveland, J. & Tuomanen, E. (1999). Neuroprotection by a caspase inhibitor in acute bacterial meningitis. *Nature Medicine,* **5,** 298-302.

Brooks, H. J., McConnell, M. A. & Broadbent, R. S. (2013). Microbes and the inflammatory response in necrotising enterocolitis. In: Erez, O. (ed.) Preterm Birth. INTECH. Available: http://www.intechopen.com/books/preterm-birth/microbes-and-the-inflammatory-response-in-necrotising-enterocolitis [Accessed 26/11/2013].

Burdette, J. & Santos, C. (2000). *Enterobacter sakazakii* brain abscess in the neonate: the importance of neuroradiologic imaging. *Pediatric Radiology,* **30,** 33-34.

Burgess, A. W. & Metcalf, D. (1980). The nature and action of granulocyte-macrophage colony stimulating factors. *Blood,* **56,** 947-958.

Burns, J. L., Griffith, A., Barry, J. J., Jonas, M. & Chi, E. Y. (2001). Transcytosis of gastrointestinal epithelial cells by *Escherichia coli* K1. *Pediatric research,* **49,** 30-37.

Carr, R., Modi, N. & Dore, C. (2003). G-CSF and GM-CSF for treating or preventing neonatal infections. *Cochrane Database Reviws* [Online], **3**. Available: http://onlinelibrary.wiley.com/store/10.1002/14651858.CD003066/asset/CD003066.pdf?v=1&t=hy9y8w3e&s=03e5af7ea6a146149c07702e82f95c17ffc75dd8.

Carver, T. J., Rutherford, K. M., Berriman, M., Rajandream, M.-A., Barrell, B. G. & Parkhill, J. (2005). ACT: the Artemis comparison tool. *Bioinformatics,* **21,** 3422-3423.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prere, M. F. & Forsythe, S. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *Journal of Clinical Microbiology,* **45,** 3979-3985.

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., Osaili, T., Shaker, R., Jaradat, Z. & Hartantyo, S. (2009). International survey of *Cronobacter sakazakii* and other Cronobacter spp. in follow up formulas and infant foods. *International Journal of Food Microbiology,* **136,** 185-188.

Chokshi, N. K., Guner, Y. S., Hunter, C. J., Upperman, J. S., Grishin, A. & Ford, H. R. (2008). The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. *Seminars in Perinatology,* **32,** 92-99.

Cohen, G. (1997). Caspases: the executioners of apoptosis. *Biochemical Journal,* **326,** 1-16.

Cohen, J. J., Duke, R. C., Fadok, V. A. & Sellins, K. S. (1992). Apoptosis and programmed cell death in immunity. *Annual Review of Immunology,* **10,** 267-293.

Coignard, B., Vaillant, V., Vincent, J., Leflèche, A., Mariani-Kurkdjian, P., Bernet, C., L'Hériteau, F., Sénéchal, H., Grimont, P. & Bingen, E. (2006). Infections sévères à *Enterobacter sakazakii* chez des nouveau-nés ayant consommé une préparation en poudre pour nourrissons, France, octobre-décembre 2004. *Bulletin Epidémiologique Hebdomadaire (INVS)*, 10-13.

Commission, C. A. (2008). Code of hygienic practice for powdered formulae for infants and young children. *CAC/RCP*, 1-29.

Couper, K. N., Blount, D. G. & Riley, E. M. (2008). IL-10: the master regulator of immunity to infection. *The Journal of Immunology, 180,* 5771-5777.

Cox, G. M., Harrison, T. S., McDade, H. C., Taborda, C. P., Heinrich, G., Casadevall, A. & Perfect, J. R. (2003). Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infection and Immunity, 71,* 173-180.

Cruz-Córdova, A., Rocha-Ramírez, L. M., Ochoa, S. A., Gónzalez-Pedrajo, B., Espinosa, N., Eslava, C., Hernández-Chiñas, U., Mendoza-Hernández, G., Rodríguez-Leviz, A., Valencia-Mayoral, P., Sadowinski-Pine, S., Hernández-Castro, R., Estrada-García, I., Muñoz-Hernández, O., Rosas, I. & Xicohtencatl-Cortes, J. (2012). Flagella from Five *Cronobacter* Species Induce Pro-Inflammatory Cytokines in Macrophage Derivatives from Human Monocytes. *PLoS One, 7,* e52091.

Dawson, K. G., Emerson, J. C. & Burns, J. L. (1999). Fifteen years of experience with bacterial meningitis. *The Pediatric Infectious Disease Journal, 18,* 816-822.

Deghmane, A.-E., Veckerlé, C., Giorgini, D., Hong, E., Ruckly, C. & Taha, M.-K. (2009). Differential modulation of TNF-α–induced apoptosis by *Neisseria meningitidis*. *PLoS Pathogens, 5,* e1000405.

Deli, M. A., Ábrahám, C. S., Kataoka, Y. & Niwa, M. (2005). Permeability studies on *in vitro* blood–brain barrier models: physiology, pathology, and pharmacology. *Cellular and Molecular Neurobiology, 25,* 59-127.

Dimmeler, S. & Zeiher, A. M. (1997). Nitric oxide and apoptosis: another paradigm for the double-edged role of nitric oxide. *Nitric Oxide: Biology and Chemistry, 1,* 275-281.

Dinarello, C. A. (1993). The biological properties of interleukin-1. *European Cytokine Network, 5,* 517-531.

Dinarello, C. A. (2007). Historical insights into cytokines. *European Journal of Immunology, 37,* 34-45.

Doran, T. I. (1999). The role of *Citrobacter* in clinical disease of children: review. *Clinical Infectious Diseases, 28,* 384-394.

Dulkerian, S. J., Kilpatrick, L., Costarino Jr, A. T., McCawley, L., Fein, J., Corcoran, L., Zirin, S. & Harris, M. C. (1995). Cytokine elevations in infants with bacterial and aseptic meningitis. *The Journal of Pediatrics, 126,* 872-876.

Eisenhauer, P. B., Chaturvedi, P., Fine, R. E., Ritchie, A. J., Pober, J. S., Cleary, T. G. & Newburg, D. S. (2001). Tumor necrosis factor alpha increases human cerebral endothelial cell Gb3 and sensitivity to Shiga toxin. *Infection and Immunity, 69,* 1889-1894.

Emami, C. N., Mittal, R., Wang, L., Ford, H. R. & Prasadarao, N. V. (2011). Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by *Cronobacter sakazakii*. *The Journal of Immunology, 186,* 7067-7079.

Emami, C. N., Mittal, R., Wang, L., Ford, H. R. & Prasadarao, N. V. (2012). Role of neutrophils and macrophages in the pathogenesis of necrotizing enterocolitis caused by *Cronobacter sakazakii*. *Journal of Surgical Research, 172,* 18-28.

Emery, C. L. & Weymouth, L. A. (1997). Detection and clinical significance of extended-spectrum beta-lactamases in a tertiary-care medical center. *Journal of Clinical Microbiology,* **35,** 2061-2067.

Epstein, F. H., Gabay, C. & Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *New England Journal of Medicine,* **340,** 448-454.

Ernst, R. K., Guina, T. & Miller, S. I. (1999). How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. *Journal of Infectious Diseases,* **179,** S326-S330.

FAO/WHO (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae. Available: https://http://www.google.com/url?q=http://www.fao.org/fileadmin/templates/agns/pdf/jemra/mra15_sakazaki.pdf&sa=U&ei=mJ4zVIr9A6nz8AGGsYH4Ag&ved=0CAUQFjAA&client=internal-uds-cse&usg=AFQjCNFNr3-jvEhr8Zsm8y-V8gk9ZY_RuQ [Accessed 07/10/2014].

Faraldo-Gómez, J. D. & Sansom, M. S. P. (2003). Acquisition of siderophores in gram-negative bacteria. *Nature Reviews Molecular Cell Biology,* **4,** 105-116.

Farber, J. M. & Forsythe, S. J. (eds.), (2008). *Enterobacter sakazakii,* Washington, USA: American Society for Microbiology.

Farmer, J. J., Asbury, M., Hickman, F. & Brenner, D. O. N. J. (1980). *Enterobacter sakazakii*: a new species of "*Enterobacteriaceae*" isolated from clinical specimens. *International Journal of Systematic Bacteriology,* **30,** 569-584.

Farr, S. B., Arnosti, D. N., Chamberlin, M. J. & Ames, B. N. (1989). An apaH mutation causes AppppA to accumulate and affects motility and catabolite repression in *Escherichia coli*. *Proceedings of the National Academy of Sciences,* **86,** 5010-5014.

Finlay, B. B. & Falkow, S. (1990). *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. *Journal of Infectious Diseases,* **162,** 1096-1106.

Firestein, G. S., Budd, R., Gabriel, S. E., O'Dell, J. R. & McInnes, I. B. (2012). Cytokines. *Kelley's Textbook of Rheumatology.* **8** ed., 367-377. Oxford, UK: Elsevier Health Sciences.

Forsythe, S. J. (2005). *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. *Maternal & Child Nutrition,* **1,** 44-50.

Forsythe, S. J., Dickins, B. & Jolley, K. A. (2014). *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. *BMC Genomics* **15,** 1121.

Fotakis, G. & Timbrell, J. A. (2006). *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters,* **160,** 171-177.

Franchi, L., Amer, A., Body-Malapel, M., Kanneganti, T.-D., Özören, N., Jagirdar, R., Inohara, N., Vandenabeele, P., Bertin, J. & Coyle, A. (2006). Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in *salmonella*-infected macrophages. *Nature Immunology,* **7,** 576-582.

Franco, A., Hu, L., Grim, C., Gopinath, G., Sathyamoorthy, V., Jarvis, K., Lee, C., Sadowski, J., Kim, J. & Kothary, M. (2011a). Characterization of putative virulence genes on the related RepFIB plasmids harbored by *Cronobacter* spp. *Applied and Environmental Microbiology,* **77,** 3255-3267.

Franco, A., Kothary, M., Gopinath, G., Jarvis, K., Grim, C., Hu, L., Datta, A., McCardell, B. & Tall, B. (2011b). *Cpa*, the outer membrane protease of *Cronobacter sakazakii*, activates plasminogen and mediates resistance to serum bactericidal activity. *Infection and Immunity,* **79,** 1578-1587.

Friedemann, M. (2009). Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *European Journal of Clinical Microbiology & Infectious Diseases, 28,* 1297-1304.

Galanakis, E., Di Cello, F., Paul-Satyaseela, M. & Kim, K. S. (2006). *Escherichia coli* K1 induces IL-8 expression in human brain microvascular endothelial cells. *European Cytokine Network, 17,* 260-265.

Gao, L.-Y. & Kwaik, Y. A. (2000). The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends in Microbiology, 8,* 306-313.

García Véscovi, E., Ayala, M., Di Cera, E. & Groisman, E. (1997). Characterization of the bacterial sensor protein PhoQ. *Journal of Biological Chemistry, 272,* 1440-1443.

García Véscovi, E., Soncini, F. C. & Groisman, E. A. (1996). Mg2+ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell, 84,* 165-174.

Gaschignard, J., Levy, C., Romain, O., Cohen, R., Bingen, E., Aujard, Y. & Boileau, P. (2011). Neonatal bacterial meningitis: 444 cases in 7 years. *The Pediatric Infectious Disease Journal, 30,* 212-217.

Giri, C. P., Shima, K., Tall, B. D., Curtis, S., Sathyamoorthy, V., Hanisch, B., Kim, K. S. & Kopecko, D. J. (2011). *Cronobacter* spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells. *Microbial Pathogenesis, 52,* 140–147.

Gottesman, S., Trisler, P. & Torres-Cabassa, A. (1985). Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. *Journal of Bacteriology, 162,* 1111-1119.

Grabenstein, J. P., Fukuto, H. S., Palmer, L. E. & Bliska, J. B. (2006). Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. *Infection and Immunity, 74,* 3727-3741.

Grabenstein, J. P., Marceau, M., Pujol, C., Simonet, M. & Bliska, J. B. (2004). The response regulator PhoP of *Yersinia pseudotuberculosis* is important for replication in macrophages and for virulence. *Infection and Immunity, 72,* 4973-4984.

Grandgirard, D. & Leib, S. L. (2010). Meningitis in neonates: bench to bedside. *Clinics in Perinatology, 37,* 655-676.

Grim, C., Kothary, M., Gopinath, G., Jarvis, K., Beaubrun, J. J.-G., McClelland, M., Tall, B. & Franco, A. (2012). Identification and characterization of *Cronobacter* iron acquisition systems. *Applied and Environmental Microbiology, 78,* 6035-6050.

Grimwood, K., Anderson, P., Anderson, V., Tan, L. & Nolan, T. (2000). Twelve year outcomes following bacterial meningitis: further evidence for persisting effects. *Archives of Disease in Childhood, 83,* 111-116.

Gross, S. S. & Wolin, M. (1995). Nitric oxide: pathophysiological mechanisms. *Annual Review of Physiology, 57,* 737-769.

Gunn, J. S., Hohmann, E. L. & Miller, S. I. (1996). Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP. *Journal of Bacteriology, 178,* 6369-6373.

Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998). PmrA–PmrB- regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Molecular Microbiology, 27,* 1171-1182.

Hackam, D. J., Upperman, J. S., Grishin, A. & Ford, H. R. (2005). Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. Seminars in Pediatric Surgery. Elsevier, 49-57.

Haiko, J., Suomalainen, M., Ojala, T., Lähteenmäki, K. & Korhonen, T. K. (2009). Invited review: Breaking barriers-attack on innate immune defences by omptin surface proteases of enterobacterial pathogens. *Innate Immunity,* **15,** 67-80.

Haiko, J. & Westerlund-Wikström, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology,* **2,** 1242-1267.

Halstensen, A., Ceska, M., Brandtzaeg, P., Redl, H., Naess, A. & Waage, A. (1993). Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. *Journal of Infectious Diseases,* **167,** 471-475.

Hamilton, J. A. (2002). GM-CSF in inflammation and autoimmunity. *Trends in Immunology,* **23,** 403-408.

Hanke, M. L. & Kielian, T. (2011). Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. *Clinical Science,* **121,** 367-387.

Hariri, S., Joseph, S. & Forsythe, S. J. (2013). *Cronobacter sakazakii* ST4 strains and neonatal meningitis, United States. *Emerging Infectious Diseases,* **19,** 175.

Hartmann, I., Carranza, P., Lehner, A., Stephan, R., Eberl, L. & Riedel, K. (2010). Genes involved in *Cronobacter sakazakii* biofilm formation. *Applied and Environmental Microbiology,* **76,** 2251-2261.

Henry, M. C. W. & Lawrence Moss, R. (2010). Necrotizing Enterocolitis. *In:* Ostlie, D. J. (ed.) *Ashcraft's Pediatric Surgery.* **5** ed., 439-455. Philadelphia: W.B. Saunders.

Himelright, I., Harris, E., Lorch, V., Anderson, M., Jones, T., Craig, A., Kuehnert, M., Forster, T., Arduino, M. & Jensen, B. (2002). *Enterobacter sakazakii* infections associated with the use of powdered infant formula-Tennessee, 2001. *Morbidity and Mortality Weekly Report*. CDC. **51**, 297-300. Available: http://www.cdc.gov/Mmwr/PDF/wk/mm5114.pdf [Accessed 13/10/2014].

Hirano, T., Akira, S., Taga, T. & Kishimoto, T. (1990). Biological and clinical aspects of interleukin 6. *Immunology Today,* **11,** 443-449.

Holman, R. C., Stoll, B. J., Curns, A. T., Yorita, K. L., Steiner, C. A. & Schonberger, L. B. (2006). Necrotising enterocolitis hospitalisations among neonates in the United States. *Paediatric and Perinatal Epidemiology,* **20,** 498-506.

Holý, O. & Forsythe, S. (2014). *Cronobacter* spp. as emerging causes of healthcare-associated infection. *Journal of Hospital Infection,* **86,** 169-177.

Honko, A. N. & Mizel, S. B. (2005). Effects of flagellin on innate and adaptive immunity. *Immunologic Research,* **33,** 83-101.

Howkins, R. E., LISSNER, C. R. & SANFORD, J. P. (1991). *Enterobacter sakazakii* bacteremia in an adult. *Southern Medical Journal,* **84,** 793.

Huang, S.-H., Chen, Y.-H., Fu, Q., Stins, M., Wang, Y., Wass, C. & Kim, K. S. (1999). Identification and characterization of an *Escherichia coli* invasion gene locus, *ibeB*, required for penetration of brain microvascular endothelial cells. *Infection and Immunity,* **67,** 2103-2109.

Hughes, C., Phillips, R. & Roberts, A. (1982). Serum resistance among *Escherichia coli* strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin, and antibiotic resistance determinants. *Infection and Immunity,* **35,** 270-275.

Hunter, C. & Bean, J. (2013). *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *Journal of Perinatology,* **33,** 581-585.

Hunter, C. J., Singamsetty, V. K., Chokshi, N. K., Boyle, P., Camerini, V., Grishin, A. V., Upperman, J. S., Ford, H. R. & Prasadarao, N. V. (2008). *Enterobacter sakazakii* enhances epithelial cell injury by inducing

apoptosis in a rat model of necrotizing enterocolitis. *Journal of Infectious Diseases,* **198,** 586.

Hunter, C. J., Williams, M., Petrosyan, M., Guner, Y., Mittal, R., Mock, D., Upperman, J. S., Ford, H. R. & Prasadarao, N. V. (2009). *Lactobacillus bulgaricus* prevents intestinal epithelial cell injury caused by *Enterobacter sakazakii*-induced nitric oxide both *in vitro* and in the newborn rat model of necrotizing enterocolitis. *Infection and Immunity,* **77,** 1031-1043.

Iben, S. & Rodriguez, R. (2011). Neonatal Necrotizing Enterocolitis. *Pediatric Gastrointestinal and Liver Disease.* **4** ed., 512-520. Saint Louis: W.B. Saunders.

ICMSF (2002). Selection of cases and attribute plans. *Micro-Organisms in Foods Vol. 7 Microbiological Testing in Food Safety Management.* Chapter 8. New York: Kluwer Academic/Plenum

Ismail, T. M., Hart, C. A. & McLennan, A. G. (2003). Regulation of dinucleoside polyphosphate pools by the YgdP and ApaH hydrolases is essential for the ability of *Salmonella enterica* serovar typhimurium to invade cultured mammalian cells. *Journal of Biological Chemistry,* **278,** 32602-32607.

Iversen, C. & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology,* **14,** 443-454.

Iversen, C. & Forsythe, S. (2004). Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. *Food Microbiology,* **21,** 771-777.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii,* comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **7,** 64.

Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **58,** 1442-1447.

Jackson, E., Sonbol, H., Masood, N. & Forsythe, S. (2014). Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *Cronobacter helveticus*, *Cronobacter pulveris*, and C*ronobacter zurichensis*. *Food Microbiology,* **44,** 226-235.

Jarvis, C. (2005). Fatal *Enterobacter sakazakii* infection associated with powdered infant formula in a neonatal intensive care unit in New Zealand. *American Journal of Infection Control,* **33,** e19.

Jersmann, H. P., Hii, C. S., Ferrante, J. V. & Ferrante, A. (2001). Bacterial lipopolysaccharide and tumor necrosis factor alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-κB and p38 mitogen-activated protein kinase signaling pathways. *Infection and Immunity,* **69,** 1273-1279.

Jiménez, E. B. & Giménez, C. R. (1982). Septic shock due to *Enterobacter sakazakii*. *Clinical Microbiology Newsletter,* **4,** 30.

Joker, R., Norholm, T. & Siboni, K. (1965). A case of neonatal meningitis caused by a yellow *Enterobacter*. *Danish Medical Bulletin*, **12,** 128.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J. & Forsythe, S. J. (2012a). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *International Journal of Systematic and Evolutionary Microbiology*, **62,** 1277-1283.

Joseph, S., Desai, P., Ji, Y., Cummings, C. A., Shih, R., Degoricija, L., Rico, A., Brzoska, P., Hamby, S. E., Masood, N., Hariri, S., Sonbol, H., Chuzhanova, N., McClelland, M., Furtado, M. R. & Forsythe, S. J. (2012b). Comparative analysis of genome sequences covering the seven *Cronobacter* species. *PLoS One,* **7,** e49455.

Joseph, S. & Forsythe, S. J. (2011). Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerging Infectious Diseases,* **17,** 1713–1715.

Joseph, S. & Forsythe, S. J. (2012c). Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by multilocus sequence typing and analysis. *Frontiers in Microbiology,* **3,** 1-11.

Joseph, S., Hariri, S., Masood, N. & Forsythe, S. (2013). Sialic acid utilization by *Cronobacter sakazakii*. *Microbial Informatics and Experimentation,* **3,** 1-11.

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M. & Forsythe, S. (2012d). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. *Journal of Clinical Microbiology,* **50,** 3031-3039.

Kastenbauer, S., Koedel, U., Weih, F., Ziegler-Heitbrock, L. & Pfister, H.-W. (2004). Protective role of NF-κB1 (p50) in experimental pneumococcal meningitis. *European Journal of Pharmacology,* **498,** 315-318.

Kawai, T. & Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity,* **34,** 637-650.

Keelan, J., Blumenstein, M., Helliwell, R., Sato, T., Marvin, K. & Mitchell, M. (2003). Cytokines, prostaglandins and parturition—a review. *Placenta,* **24,** S33-S46.

Kielian, T., McMahon, M., Bearden, E. D., Baldwin, A. C., Drew, P. D. & Esen, N. (2004). *S. aureus*- dependent microglial activation is selectively attenuated by the cyclopentenone prostaglandin 15- deoxy- Δ12, 14- prostaglandin J2 (15d- PGJ2). *Journal of Neurochemistry,* **90,** 1163-1172.

Kim, H., Ryu, J.-H. & Beuchat, L. R. (2006). Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. *Applied and Environmental Microbiology,* **72,** 5846-5856.

Kim, K., Kim, K. P., Choi, J., Lim, J. A., Lee, J., Hwang, S. & Ryu, S. (2010). Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. *Applied and Environmental Microbiology,* **76,** 5188-5198.

Kim, K. P. & Loessner, M. J. (2008). *Enterobacter sakazakii* invasion in human intestinal Caco-2 cells requires the host cell cytoskeleton and is enhanced by disruption of tight junction. *Infection and Immunity,* **76,** 562-570.

Kim, K. S. (2003). Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury. *Nature Reviews Neuroscience,* **4,** 376-385.

Kim, Y. S., Kennedy, S. & Tauber, M. G. (1995). Toxicity of *Streptococcus pneumoniae* in neurons, astrocytes, and microglia *in vitro*. *Journal of Infectious Diseases,* **171,** 1363-1368.

Kishimoto, T., Akira, S. & Taga, T. (1992). Interleukin-6 and its receptor: a paradigm for cytokines. *Science,* **258,** 593-597.

Koedel, U., Bayerlein, I., Paul, R., Sporer, B. & Pfister, H. (2000). Pharmacologic interference with NF-κB activation attenuates central

nervous system complications in experimental pneumococcal meningitis. *Journal of Infectious Diseases,* **182,** 1437-1445.

Köster, W. (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Research in Microbiology,* **152,** 291-301.

Krebs, V. L. J., Okay, T. S., Okay, Y. & Vaz, F. A. C. (2005). Tumor necrosis factor-alpha, interleukin-1beta and interleukin-6 in the cerebrospinal fluid of newborn with meningitis. *Arquivos de Neuro-Psiquiatria,* **63,** 7-13.

Kröncke, K.-D., Fehsel, K. & Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection—how, why, when, and where? *Nitric Oxide: Biology and Chemistry,* **1,** 107-120.

Kronfol, Z. & Remick, D. G. (2000). Cytokines and the brain: implications for clinical psychiatry. *American Journal of Psychiatry,* **157,** 683-694.

Krzymińska, S., Mokracka, J., Koczura, R. & Kaznowski, A. (2009). Cytotoxic activity of *Enterobacter cloacae* human isolates. *FEMS Immunology & Medical Microbiology,* **56,** 248-252.

Kucerova, E., Clifton, S. W., Xia, X. Q., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K., Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W. E., Hallsworth-Pepin, K., Wilson, R. K., McClelland, M. & Forsythe, S. J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLoS One,* **5,** e9556.

Kukkonen, M. & Korhonen, T. K. (2004). The omptin family of enterobacterial surface proteases/adhesins: from housekeeping in *Escherichia coli* to systemic spread of *Yersinia pestis*. *International Journal of Medical Microbiology,* **294,** 7-14.

Kukkonen, M., Lähteenmäki, K., Suomalainen, M., Kalkkinen, N., Emödy, L., Lång, H. & Korhonen, T. K. (2001). Protein regions important for plasminogen activation and inactivation of α2-antiplasmin in the surface protease *Pla* of *Yersinia pestis*. *Molecular Microbiology,* **40,** 1097-1111.

Kumar, C. G. & Anand, S. (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology,* **42,** 9-27.

Lähteenmäki, K., Edelman, S. & Korhonen, T. K. (2005). Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends in Microbiology,* **13,** 79-85.

Lai, K. K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. *Medicine,* **80,** 113-122.

Leib, S. L. & Täuber, M. G. (1999). Pathogenesis of bacterial meningitis. *Infectious Disease Clinics of North America,* **13,** 527-548.

Leist, T. P., Frei, K., Kam-Hansen, S., Zinkernagel, R. & Fontana, A. (1988). Tumor necrosis factor alpha in cerebrospinal fluid during bacterial, but not viral, meningitis. Evaluation in murine model infections and in patients. *The Journal of Experimental Medicine,* **167,** 1743-1748.

Li, G., Laturnus, C., Ewers, C. & Wieler, L. H. (2005). Identification of genes required for avian *Escherichia coli* septicemia by signature-tagged mutagenesis. *Infection and Immunity,* **73,** 2818-2827.

Liu, B., Wang, K., Gao, H. M., Mandavilli, B., Wang, J. Y. & Hong, J. S. (2001). Molecular consequences of activated microglia in the brain: overactivation induces apoptosis. *Journal of Neurochemistry,* **77,** 182-189.

Liu, L., Johnson, H. L., Cousens, S., Perin, J., Scott, S., Lawn, J. E., Rudan, I., Campbell, H., Cibulskis, R. & Li, M. (2012a). Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *The Lancet,* **379,** 2151-2161.

Liu, Q., Mittal, R., Emami, C. N., Iversen, C., Ford, H. R. & Prasadarao, N. V. (2012b). Human isolates of *Cronobacter sakazakii* bind efficiently to

intestinal epithelial cells *in vitro* to induce monolayer permeability and apoptosis. *Journal of Surgical Research,* **176,** 437-447.

Liu, S. & Kielian, T. (2009). Microglial activation by *Citrobacter koseri* is mediated by TLR4-and MyD88-dependent pathways. *The Journal of Immunology,* **183,** 5537-5547.

Mange, J. P., Stephan, R., Borel, N., Wild, P., Kim, K. S., Pospischil, A. & Lehner, A. (2006). Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. *BMC Microbiology,* **6,** 58.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology,* **25,** 677-686.

Marshall, K. C. (1992). Biofilms: an overview of bacterial adhesion, activity, and control at surfaces. Control of biofilm formation awaits the development of a method to prevent bacterial adhesion. *ASM American Society for Microbiology News,* **58,** 202-207.

Mayer, B. & Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends in Biochemical Sciences,* **22,** 477-481.

McCallum, K. L. & Whitfield, C. (1991). The rcsA gene of *Klebsiella pneumoniae* O1: K20 is involved in expression of the serotype-specific K (capsular) antigen. *Infection and Immunity,* **59,** 494-502.

Miajlovic, H., Cooke, N. M., Moran, G. P., Rogers, T. R. & Smith, S. G. (2014). Response of extraintestinal pathogenic *Escherichia coli* to human serum reveals a protective role for Rcs-regulated exopolysaccharide colanic acid. *Infection and Immunity,* **82,** 298-305.

Miajlovic, H. & Smith, S. G. (2014). Bacterial self- defence: how *Escherichia coli* evades serum killing. *FEMS Microbiology Letters,* **354,** 1-9.

Miethke, M. & Marahiel, M. A. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Reviews,* **71,** 413-451.

Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP -phoQ*) controls *Salmonella typhimurium* virulence. *Proceedings of the National Academy of Sciences,* **86,** 5054-5058.

Mitchell, D., Yong, M., Schroder, W., Black, M., Tirrell, M. & Olive, C. (2010). Dual stimulation of MyD88-dependent Toll-like receptors induces synergistically enhanced production of inflammatory cytokines in murine bone marrow-derived dendritic cells. *Journal of Infectious Diseases,* **202,** 318-329.

Mohan Nair, M. K., Venkitanarayanan, K., Silbart, L. K. & Kim, K. S. (2009). Outer membrane protein A (OmpA) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. *Foodborne Pathogens and Disease,* **6,** 495-501.

Mook-Kanamori, B. B., Geldhoff, M., van der Poll, T. & van de Beek, D. (2011). Pathogenesis and pathophysiology of pneumococcal meningitis. *Clinical Microbiology Reviews,* **24,** 557-591.

Moresco, E. M. Y., LaVine, D. & Beutler, B. (2011). Toll-like receptors. *Current Biology,* **21,** R488-R493.

Mramba, F., Broce, A. & Zurek, L. (2007). Vector competence of stable flies, Stomoxys calcitrans L.(Diptera: Muscidae), for *Enterobacter sakazakii*. *Journal of Vector Ecology,* **32,** 134-139.

Muytjens, H., Zanen, H., Sonderkamp, H., Kollee, L., Wachsmuth, I. K. & Farmer, J. (1983). Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. *Journal of Clinical Microbiology,* **18,** 115-120.

Muytjens, H. L., Roelofs-Willemse, H. & Jaspar, G. (1988). Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *Journal of Clinical Microbiology, 26,* 743-746.

Nadal, D., Leppert, D., Frei, K., Gallo, P., Lamche, H. & Fontana, A. (1989). Tumour necrosis factor-alpha in infectious meningitis. *Archives of Disease in Childhood, 64,* 1274-1279.

Nazarowec-White, M. & Farber, J. (2003). Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. *Letters in Applied Microbiology, 24,* 9-13.

Neilands, J. (1981). Iron absorption and transport in microorganisms. *Annual Review of Nutrition, 1,* 27-46.

Nizet, V., Kim, K., Stins, M., Jonas, M., Chi, E. Y., Nguyen, D. & Rubens, C. E. (1997). Invasion of brain microvascular endothelial cells by group B *streptococci*. *Infection and Immunity, 65,* 5074-5081.

O'rahilly, R. & Möller, F. (1986). The meninges in human development. *Journal of Neuropathology & Experimental Neurology, 45,* 588-608.

Ockenhouse, C. F., Tegoshi, T., Maeno, Y., Benjamin, C., Ho, M., Kan, K. E., Thway, Y., Win, K., Aikawa, M. & Lobb, R. (1992). Human vascular endothelial cell adhesion receptors for *Plasmodium falciparum*-infected erythrocytes: roles for endothelial leukocyte adhesion molecule 1 and vascular cell adhesion molecule 1. *The Journal of Experimental Medicine, 176,* 1183-1189.

Odio, C. M., Faingezicht, I., Paris, M., Nassar, M., Baltodano, A., Rogers, J., Sáez-Llorens, X., Olsen, K. D. & McCragken Jr, G. H. (1991). The beneficial effects of early dexamethasone administration in infants and children with bacterial meningitis. *New England Journal of Medicine, 324,* 1525-1531.

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H. & Kanehisa, M. (1999). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research, 27,* 29-34.

Ohga, S., Aoki, T., Okada, K., Akeda, H., Fujioka, K., Ohshima, A., Mori, T., Minamishima, I. & Ueda, K. (1994). Cerebrospinal fluid concentrations of interleukin-1 beta, tumour necrosis factor-alpha, and interferon gamma in bacterial meningitis. *Archives of Disease in Childhood, 70,* 123-125.

Oyston, P. C., Dorrell, N., Williams, K., Li, S.-R., Green, M., Titball, R. W. & Wren, B. W. (2000). The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*. *Infection and Immunity, 68,* 3419-3425.

Pagotto, F. J., Nazarowec-White, M., Bidawid, S. & Farber, J. M. (2003). *Enterobacter sakazakii*: infectivity and enterotoxin production *in vitro* and *in vivo*. *Journal of Food Protection, 66,* 370-375.

París, M. M., Hickey, S. M., Trujillo, M., Ahmed, A., Olsen, K. & McCracken, G. H. (1997). The effect of interleukin-10 on meningeal inflammation in experimental bacterial meningitis. *Journal of Infectious Diseases, 176,* 1239-1246.

Parm, Ü., Metsvaht, T., Sepp, E., Ilmoja, M.-L., Pisarev, H., Pauskar, M. & Lutsar, I. (2011). Risk factors associated with gut and nasopharyngeal colonization by common Gram-negative species and yeasts in neonatal intensive care units patients. *Early Human Development, 87,* 391-399.

Parrow, N. L., Fleming, R. E. & Minnick, M. F. (2013). Sequestration and Scavenging of Iron in Infection. *Infection and Immunity, 81,* 3503-3514.

Parthasarathy, G. & Philipp, M. T. (2012). Review: apoptotic mechanisms in bacterial infections of the central nervous system. *Frontiers in Immunology, 3.*

Patrick, M. E., Mahon, B. E., Greene, S. A., Rounds, J., Cronquist, A., Wymore, K., Boothe, E., Lathrop, S., Palmer, A. & Bowen, A. (2014).

Incidence of *Cronobacter* spp. Infections, United States, 2003-2009. *Emerging Infectious Diseases,* **20,** 1536-1539.

Pavcnik-Arnol, M., Hojker, S. & Derganc, M. (2007). Lipopolysaccharide-binding protein, lipopolysaccharide, and soluble CD14 in sepsis of critically ill neonates and children. *Intensive Care Medicine,* **33,** 1025-1032.

Peng, L., Luo, W.-Y., Zhao, T., Wan, C.-S., Jiang, Y., Chi, F., Zhao, W., Cao, H. & Huang, S.-H. (2012). Polyphosphate kinase 1 is required for the pathogenesis process of meningitic *Escherichia coli* K1 (RS218). *Future Microbiology,* **7,** 411-423.

Petrosyan, M., Guner, Y. S., Williams, M., Grishin, A. & Ford, H. R. (2009). Current concepts regarding the pathogenesis of necrotizing enterocolitis. *Pediatric Surgery International,* **25,** 309-318.

Pfister, H.-W., Fontana, A., Täuber, M. G., Tomasz, A. & Scheld, W. M. (1994). Mechanisms of brain injury in bacterial meningitis: workshop summary. *Clinical Infectious Diseases,* **19,** 463-479.

Polazzi, E. & Monti, B. (2010). Microglia and neuroprotection: from *in vitro* studies to therapeutic applications. *Progress in Neurobiology,* **92,** 293-315.

Pollara, G., Savy, L., Cropley, I. & Hopkins, S. (2011). *Citrobacter koseri* meningitis: Another freediving risk? *Journal of Infection,* **62,** 101-103.

Prasad, K., Rai, N. K. & Kumar, A. (2012). Use of corticosteroids and other adjunct therapies for acute bacterial meningitis in adults. *Current Infectious Disease Reports,* **14,** 445-453.

Pujol, C., Klein, K. A., Romanov, G. A., Palmer, L. E., Cirota, C., Zhao, Z. & Bliska, J. B. (2009). *Yersinia pestis* can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. *Infection and Immunity,* **77,** 2251-2261.

Ramegowda, B., Samuel, J. E. & Tesh, V. L. (1999). Interaction of Shiga toxins with human brain microvascular endothelial cells: cytokines as sensitizing agents. *Journal of Infectious Diseases,* **180,** 1205-1213.

Ramu, P., Tanskanen, R., Holmberg, M., Lahteenmaki, K., Korhonen, T. K. & Meri, S. (2007). The surface protease *PgtE* of *Salmonella enterica* affects complement activity by proteolytically cleaving C3b, C4b and C5. *FEBS letters,* **581,** 1716-1720.

RASFF (2002). Alert notification 2002.190: *Enterobacter sakazakii* in infant formula from Germany. Available: https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationDetail&NOTIF_REFERENCE=2002.190# [Accessed 07/10/2014].

RASFF (2004). Alert notification 2004.658: *Entrobacter sakazakii* in infant formula. Available: https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationDetail&NOTIF_REFERENCE=2004.658 [Accessed 07/10/2014].

RASFF (2007). Alert notification 2007.452: *Enterobacter sakazakii* (present/10g) in infant formula from Switzerland via Germany. Available: https://webgate.ec.europa.eu/rasff-window/portal/?event=notificationDetail&NOTIF_REFERENCE=2007.0452 [Accessed 07/10/2014].

Rautemaa, R. & Meri, S. (1999). Complement-resistance mechanisms of bacteria. *Microbes and Infection,* **1,** 785-794.

Ray, P., Das, A., Gautam, V., Jain, N., Narang, A. & Sharma, M. (2007). *Enterobacter sakazakii* in infants: novel phenomenon in India. *Indian Journal of Medical Microbiology,* **25,** 408.

Ribeiro, C., Davis, P. & Jones, D. (1976). *Citrobacter koseri* meningitis in a special care baby unit. *Journal of clinical pathology,* **29,** 1094-1096.

Rose, S. (1979). Neonatal meningitis due to *Citrobacter koseri*. *Journal Perinatal Medicine,* **7,** 273-5.

References

Rubin, L. & Staddon, J. (1999). The cell biology of the blood-brain barrier. *Annual Review of Neuroscience,* **22,** 11-28.

Ruef, C. & Coleman, D. L. (1990). Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. *Review of Infectious Diseases,* **12,** 41-62.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.-A. & Barrell, B. (2000). Artemis: sequence visualization and annotation. *Bioinformatics,* **16,** 944-945.

Sansonetti, P., Arondel, J., Huerre, M., Harada, A. & Matsushima, K. (1999). Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. *Infection and Immunity,* **67,** 1471-1480.

Schairer, D. O., Chouake, J. S., Nosanchuk, J. D. & Friedman, A. J. (2012). The potential of nitric oxide releasing therapies as antimicrobial agents. *Virulence,* **3,** 271-279.

Scheld, W. M., Koedel, U., Nathan, B. & Pfister, H.-W. (2002). Pathophysiology of bacterial meningitis: mechanism (s) of neuronal injury. *Journal of Infectious Diseases,* **186,** S225-S233.

Schubert-Unkmeir, A., Sokolova, O., Panzner, U., Eigenthaler, M. & Frosch, M. (2007). Gene expression pattern in human brain endothelial cells in response to *Neisseria meningitidis*. *Infection and Immunity,* **75,** 899-914.

Schwizer, S., Tasara, T., Zurfluh, K., Stephan, R. & Lehner, A. (2013). Identification of genes involved in serum tolerance in the clinical strain *Cronobacter sakazakii* ES5. *BMC Microbiology,* **13,** 38.

Sellner, J., Täuber, M. G. & Leib, S. L. (2010). Pathogenesis and pathophysiology of bacterial CNS infections. *In:* Roos, K. L. & Tunkel, A. R. (eds.) *Handbook of clinical neurology.* 1-16. Philadelphia: PA: Elsevier.

Sharma, V., Shukla, R. K., Saxena, N., Parmar, D., Das, M. & Dhawan, A. (2009). DNA damaging potential of zinc oxide nanoparticles in human epidermal cells. *Toxicology Letters,* **185,** 211-218.

Shin, H., Lee, J.-H., Choi, Y. & Ryu, S. (2012). Complete genome sequence of the opportunistic food-borne pathogen *Cronobacter sakazakii* ES15. *Journal of Bacteriology,* **194,** 4438-4439.

Shin, S. H., Lim, Y., Lee, S. E., Yang, N. W. & Rhee, J. H. (2001). CAS agar diffusion assay for the measurement of siderophores in biological fluids. *Journal of Microbiological Methods,* **44,** 89-95.

Singamsetty, V. K., Wang, Y., Shimada, H. & Prasadarao, N. V. (2008). Outer membrane protein A expression in *Enterobacter sakazakii* is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. *Microbial Pathogenesis,* **45,** 181-191.

Snavely, M., Miller, C. & Maguire, M. (1991). The *mgtB* Mg2+ transport locus of *Salmonella typhimurium* encodes a P-type ATPase. *Journal of Biological Chemistry,* **266,** 815-823.

Sodeinde, O. A., Subrahmanyam, Y., Stark, K., Quan, T., Bao, Y. & Goguen, J. D. (1992). A surface protease and the invasive character of plague. *Science,* **258,** 1004-1007.

Sprenger, H., Rösler, A., Tonn, P., Braune, H., Huffmann, G. & Gemsa, D. (1996). Chemokines in the cerebrospinal fluid of patients with meningitis. *Clinical Immunology and Immunopathology,* **80,** 155-161.

Stoll, B. J., Hansen, N., Fanaroff, A. A. & Lemons, J. A. (2004). *Enterobacter sakazakii* is a rare cause of neonatal septicemia or meningitis in VLBW infants. *The Journal of pediatrics,* **144,** 821-823.

Suijkerbuijk, A. (2008). Gesignaleerd. *Tijdschrift voor Diergeneeskunde,* **133,** 530.

Tall, B. D., Chen, Y., Yan, Q., Gopinath, G. R., Grim, C. J., Jarvis, K. G., Fanning, S. & Lampel, K. A. (2014). *Cronobacter*: an emergent

pathogen causing meningitis to neonates through their feeds. *Science Progress*, **97,** 154-172.

Tanaka, M., Sotomatsu, A., Yoshida, T., Hirai, S. & Nishida, A. (1994). Detection of Superoxide Production by Activated Microglia Using a Sensitive and Specific Chemiluminescence Assay and Microglia- Mediated PC12h Cell Death. *Journal of Neurochemistry*, **63,** 266-270.

Täuber, M. G. & Moser, B. (1999). Cytokines and chemokines in meningeal inflammation: biology and clinical implications. *Clinical Infectious Diseases*, 1-11.

Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiological Reviews*, **47,** 46.

Townsend, S., Caubilla Barron, J., Loc-Carrillo, C. & Forsythe, S. (2007a). The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. *Food Microbiology*, **24,** 67-74.

Townsend, S., Hurrell, E. & Forsythe, S. (2008). Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. *BMC Microbiology*, **8,** 64.

Townsend, S., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J. G., Forsythe, S. & Badger, J. L. (2007b). *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. *Microbiology*, **153,** 3538-3547.

Travan, A., Pelillo, C., Donati, I., Marsich, E., Benincasa, M., Scarpa, T., Semeraro, S., Turco, G., Gennaro, R. & Paoletti, S. (2009). Non-cytotoxic silver nanoparticle-polysaccharide nanocomposites with antimicrobial activity. *Biomacromolecules*, **10,** 1429-1435.

Tunkel, A. R., Hartman, B. J., Kaplan, S. L., Kaufman, B. A., Roos, K. L., Scheld, W. M. & Whitley, R. J. (2004). Practice guidelines for the management of bacterial meningitis. *Clinical Infectious Diseases*, **39,** 1267-1284.

Tunkel, A. R. & Scheld, W. M. (1993). Pathogenesis and pathophysiology of bacterial meningitis. *Clinical Microbiology Reviews*, **6,** 118-136.

Urmenyi, A. M. C. & Franklin, A. W. (1961). Neonatal death from pigmented coliform infection. *The Lancet*, **277,** 313-315.

van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology*, **39,** 293-297.

van de Beek, D., de Gans, J., Spanjaard, L., Weisfelt, M., Reitsma, J. B. & Vermeulen, M. (2004). Clinical features and prognostic factors in adults with bacterial meningitis. *New England Journal of Medicine*, **351,** 1849-1859.

van de Beek, D., Weisfelt, M., de Gans, J., Tunkel, A. R. & Wijdicks, E. F. (2006). Drug insight: adjunctive therapies in adults with bacterial meningitis. *Nature Clinical Practice Neurology*, **2,** 504-516.

van Engeland, M., Ramaekers, F. C., Schutte, B. & Reutelingsperger, C. P. (1996). A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry*, **24,** 131-139.

van Furth, A., Roord, J. J. & Van Furth, R. (1996). Roles of proinflammatory and anti-inflammatory cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. *Infection and Immunity*, **64,** 4883.

Verma, S., Nakaoke, R., Dohgu, S. & Banks, W. A. (2006). Release of cytokines by brain endothelial cells: a polarized response to lipopolysaccharide. *Brain, Behavior, and Immunity*, **20,** 449-455.

Wen, L.-L., Chiu, C.-T., Huang, Y.-N., Chang, C.-F. & Wang, J.-Y. (2007). Rapid glia expression and release of proinflammatory cytokines in

experimental *Klebsiella pneumoniae* meningoencephalitis. *Experimental Neurology,* **205,** 270-278.

Wieler, L. H., Semmler, T., Eichhorn, I., Antao, E. M., Kinnemann, B., Geue, L., Karch, H., Guenther, S. & Bethe, A. (2011). No evidence of the Shiga toxin-producing *E. coli* O104: H4 outbreak strain or enteroaggregative *E. coli* (EAEC) found in cattle faeces in northern Germany, the hotspot of the 2011 HUS outbreak area. *Gut Pathog,* **3,** 17-26.

Wilson, M., McNab, R. & Henderson, B. (2002). *Bacterial disease mechanisms: an introduction to cellular microbiology*, Cambridge University Press.

Wu, U. I., Mai, F. D., Sheu, J. N., Chen, L. Y., Liu, Y. T., Huang, H. C. & Chang, H. M. (2011). Melatonin inhibits microglial activation, reduces pro- inflammatory cytokine levels, and rescues hippocampal neurons of adult rats with acute *Klebsiella pneumoniae* meningitis. *Journal of Pineal Research,* **50,** 159-170.

Xaio, H., Banks, W., Niehoff, M. L. & Morley, J. (2001). Effect of LPS on the permeability of the blood–brain barrier to insulin. *Brain Research,* **896,** 36-42.

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X.-F. & Achong, M. K. (1998). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *Journal of Clinical Investigation,* **101,** 311.

Yamamoto, T., Hanawa, T., Ogata, S. & Kamiya, S. (1996). Identification and characterization of the *Yersinia enterocolitica gsrA* gene, which protectively responds to intracellular stress induced by macrophage phagocytosis and to extracellular environmental stress. *Infection and Immunity,* **64,** 2980-2987.

Yoshida, T., Tanaka, M., Sotomatsu, A. & Hirai, S. (1995). Activated microglia cause superoxide-mediated release of iron from ferritin. *Neuroscience Letters,* **190,** 21-24.

Zhang, G., Gurtu, V., Kain, S. R. & Yan, G. (1997). Early detection of apoptosis using a fluorescent conjugate of annexin V. *Biotechniques,* **23,** 525-531.

Zhou, Y., Tao, J., Yu, H., Ni, J., Zeng, L., Teng, Q., Kim, K. S., Zhao, G.-P., Guo, X. & Yao, Y. (2012). Hcp family proteins secreted via the type VI secretion system coordinately regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. *Infection and Immunity,* **80,** 1243-1251.

Zlokovic, B. V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron,* **57,** 178-201.

Zychlinsky, A. & Sansonetti, P. J. (1997). Apoptosis as a proinflammatory event: what can we learn from bacteria-induced cell death? *Trends in Microbiology,* **5,** 201-204.

Appendix.1

A.1. Tissue culture experiments for *C. sakazakii* strain 1587

C. sakazakii strain 1587 (ST109) is a CSF isolate that belongs to CC4 and was associated with neonatal meningitis infection that led to brain damage. It showed the ability to attach and invade the Caco-2 cell line ($P<0.05$ and $P<0.01$ respectively; Fig.A.1-2). Moreover, it was able to translocate through the same cell line significantly ($P<0.001$) over 5 hours of incubation (Fig.A.3). Furthermore, it adhered to HBMEC cells and showed significant invasion ($P<0.001$; Fig.A.4-5). The results of these experiments suggest that this is strain has invasion potential in addition to that it is a clinical isolates. Hence, it was added to the list of strains used at the final stage of this project (Table.2.4).
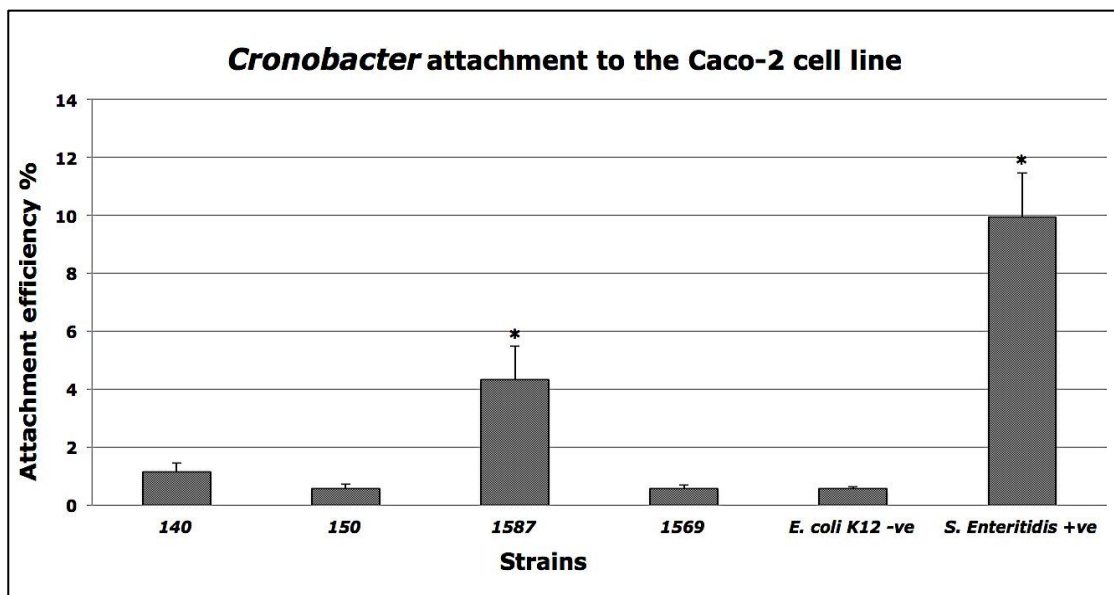
Fig.A.1 *C. sakazakii* strain 1587 attachment assay using Caco-2 cell line over 3 hours of incubation showing the difference in attachment levels among strains. The displayed data are the mean±standard deviation of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.05$).
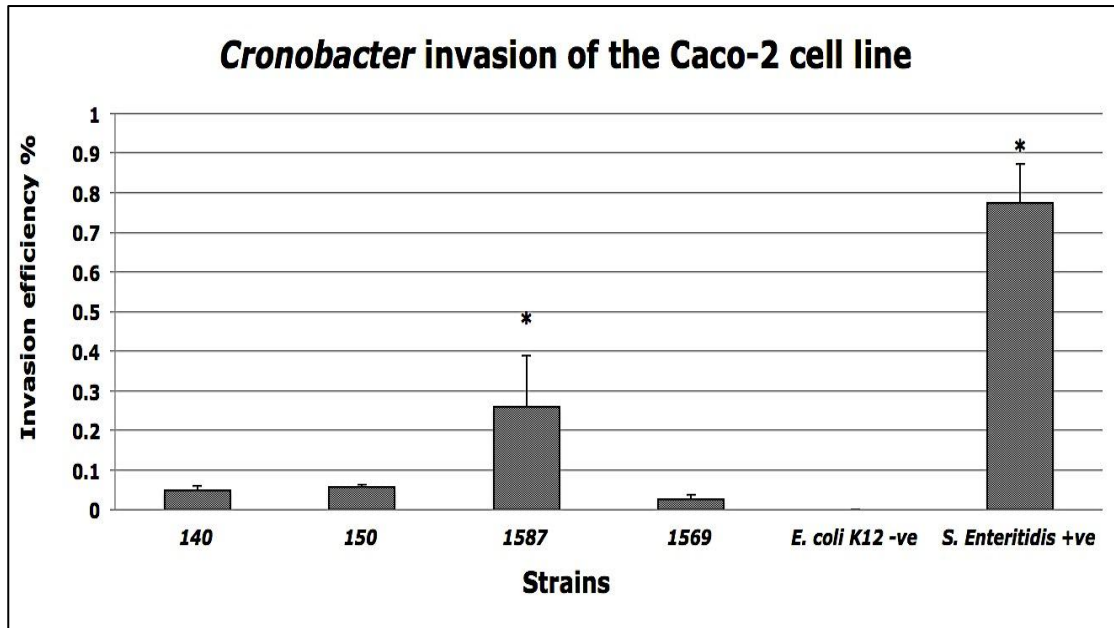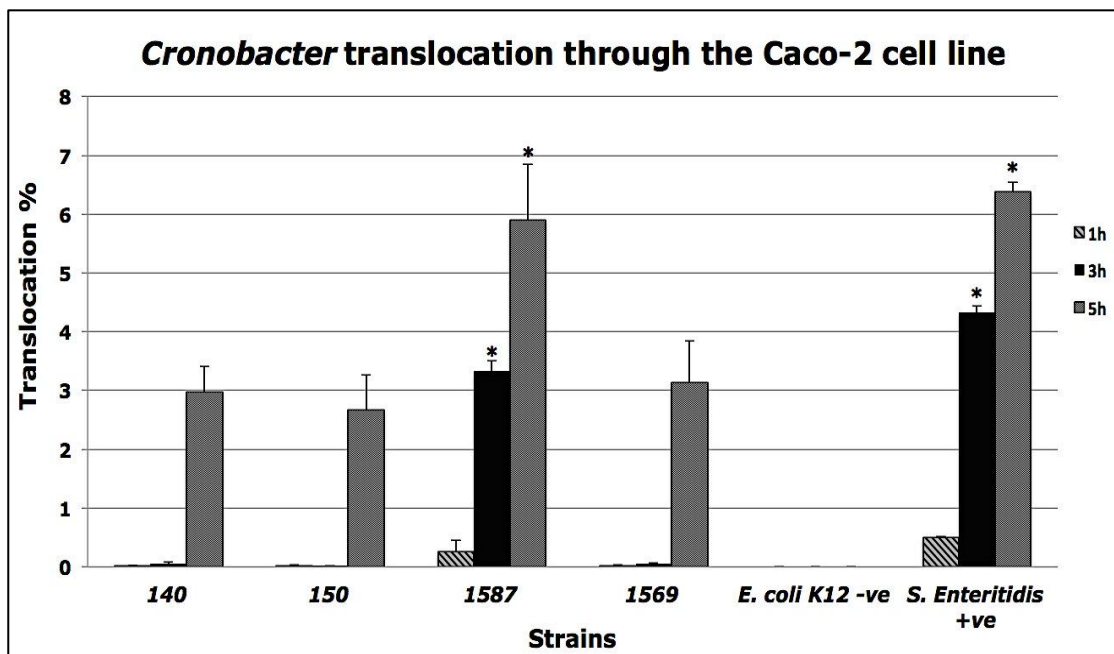
Fig.A.2 *C. sakazakii* strain 1587 invasion assay using Caco-2 cell line over 3 hours of incubation showing the difference in invasion levels among strains. The displayed data are the mean±standard deviation of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$).

Fig.A.3 *C. sakazakii* strain 1587 translocation assay using Caco-2 cell line over 5 hours of incubation showing the difference in translocation ability among strains. The displayed data are the mean±standard deviation of two independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.001$).
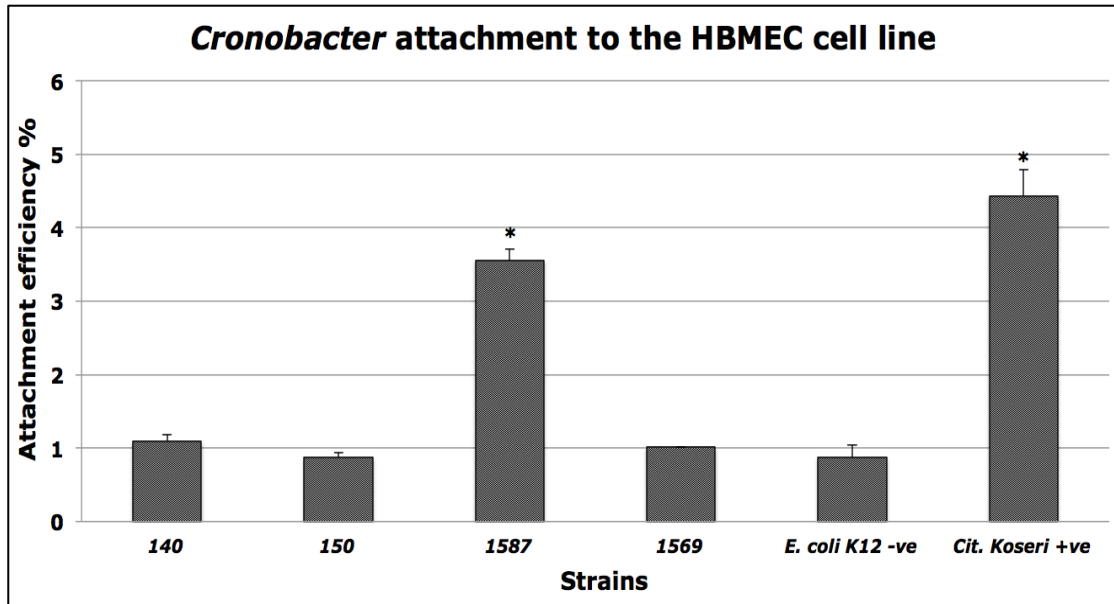
Fig.A.4 *C. sakazakii* strain 1587 attachment assay using HBMEC cell line over 3 hours of incubation showing the difference in attachment levels among strains. The displayed data are the mean±standard deviation of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.01$).
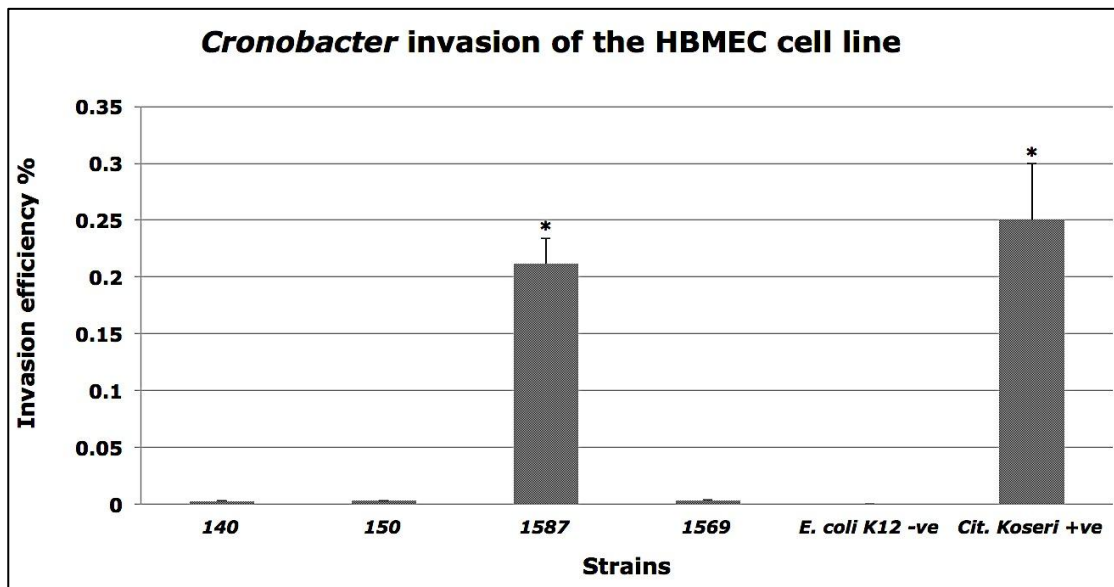

Fig.A.5 *C. sakazakii* strain 1587 invasion assay using HBMEC cell line over 3 hours of incubation showing the difference in invasion levels among strains. The displayed data are the mean±standard deviation of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*$P<0.001$).