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Neonatal exposure to pathogens: determining key virulence factors

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Declaration

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Abstract

The neonatal stage is the most critical period for infections, with mortality rate of up to 45% amongst children under five years. The genus *Cronobacter* has been involved in many outbreaks in neonatal intensive care units, with recorded meningitis, bacteraemia, and necrotizing enterocolitis (NEC). Although this genus was deeply investigated in *vitro* with different cell lines, until now, few researches have been focused on the use of H4 cell line, which is supposed to be more representative of neonatal response. Therefore, the present PhD study aimed to investigate the interaction of selected clinical isolates and the seven-type species of the genus Cronobacter, as well as one E. coli K1 isolate with H4 cells compared with Caco-2 cell line.

Physiological analyses revealed most of the strains to be motile, and capsule and biofilm producers. A link between capsule production and serum resistance was shown by some strains. Strains were examined regarding their attachment, invasion, translocation and cytotoxicity as well as for the role of host cytoskeleton in the invasion process to both cell lines. All strains, especially *C. sakazakii* ST12 (696 and 703) were significantly more adhesive to the H4 than Caco-2 cells; this sequence type has previously been associated with neonatal NEC. Importantly, this study indicated that some clinical strains were more invasive to H4 than Caco-2 cells such as *C. sakazakii* 767 (meningitis) and 701 (NECIII). Moreover, attachment and invasion of *E. coli* were higher in H4 than Caco-2 cells. High attachment and invasion is potentially linked with excessive inflammatory response and NEC development in neonates.

Most studied strains were able to translocate human cell lines, causing necrotic damage in the polarized monolayer. More importantly, translocation of blood or meningitic isolates such as *C. sakazakii* 709, *C. malonaticus* 1569, *C. turicensis* 564 was higher via H4 compared with Caco-2 cells. However, translocation of *E. coli* K1 strain 939 was similar in both cell lines. Most of the bacterial strains were cytotoxic to both cell lines and *C. sakazakii* ST3 strain 798 showed an ability to kill H4 cells up to 90-fold of blank, and about 70-fold when co-cultured with Caco-2 cells. results suggests that intact bacterial cells that able to produce new proteins while in direct contact with the host cells is essential for the cytotoxicity, indicating the potential involvement of an active secretion system in cytotoxicity.

Cytochalasin D and Colchicine mostly inhibited invasion to the H4 cells and enhanced the invasion of some strains to Caco-2 up to five-fold. Only Nocodazole significantly enhanced the invasion of some strains to H4 cell, and variably effected strains' invasion to Caco-2. Data obtained from human cytoskeleton inhibitors experiments suggested the possible strain specific role of inhibitors on both cell lines, and strains may encode different pathways for uptake, with the possible involvement of eukaryotic receptors that recognize the invading bacteria.

This study indicated that the response of the H4 cells to bacterial challenge and production of inflammatory cytokines was higher than Caco-2 cells. The H4 cells produced more IL-1-β, IL-4, IL-6, IL-8, IP-10, MCP-1, and EGF-α. Furthermore, tested strains (n=12) variably affected the expression of human Toll-like receptors (TLRs) and NF-kB subunits 1 and 2 in both cell lines, only *C. sakazakii* strain 709 upregulated expression of TLR1-4 in H4 cells and was the strongest inducer of receptor gene expression in both cell lines.

More importantly, the upregulation of NF-kB subunits 1 is more likely related to the increased inflammatory cytokine production, while no link with subunit 2. However, TLR-1 was not expressed in Caco-2 in response to these strains, which needs further investigation. The effect of the *E. coli* K1 strain 939 on the TLRs expression was varied and no specific pattern was detected, but in some cases it showed similarity to the effect of *C. sakazakii* 767 which is also linked with neonatal meningitis.

As most of the investigated bacterial strains displayed virulence factors with H4 cells closely related to their clinical pathology, the present study provides an important initial work to introduce H4 cells as a new modle for neonatal cell lines to analyse neonatal infections.

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Abbreviations

BBB: Blood brain barrier

DMEM: Dulbecco's modified Eagle's medium

EFT: Enteral feeding tube

EGF: Epidermal growth factor

Epo: Erythropoietin

FBS: Fetal bovine serum

FIIF: Fatal Infant Infection

hr: Hour.

hrs: Hours

IBD: Inflammatory bowel disease.

IEC: Intestinal epithelial cell

IFN: Interferon.

IL: Interleukin.

IL-8: Proinflammatory interleukin 8.

Info: Information

IRFs: Interferon regulatory factors

KDa Kilo Dalton

MCP-1: Monocyte chemoattractant protein 1.

MIC: Minimum inhibitory concentration

MLSA Multilocus sequence analysis

MLST Multilocus sequence typing

MOI: Multiplicity of Infection.

NA: Not applicable.

NEC: Necrotizing enterocolitis

NF-κB: Nuclear factor-kappaB

NICU Neonatal intensive care unit

NMEC Neonatal meningitis *Escherichia coli*

NTU Nottingham Trent University

OD Optical density

O-LPS Oligo-lipopolysaccharide

OM Outer membrane

OMP Outer membrane proteins

ON: Overnight

PBS Phosphate buffered saline

PCA Plate count agar

PCR Polymerase chain reaction

PFGE Pulsed field gel electrophoresis

Pg/ml Picogram/millilitre.

PIF Powdered infant formula

ROS: Reactive oxygen Species

rRNA Ribosomal ribonucleic acid

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SNP Single nucleotide polymorphism

ST: Sequence type.

TEMED N,N,N'N'-tetramethyl-ethylenedamine

TGF: Transforming growth factor

TGF-β, Transforming growth factor-beta

TNF:α Tumour necrosis factor alpha.

TS: Test sensitivity.

TSA Tryptic soy agar

TSB Tryptic soy broth

TsFHI: Temperature-sensitive fetal human intestinal cells

UV Ultraviolet

VRBGA Violet red bile glucose agar

VRBLA Violet red bile lactose agar

WGS Whole genome sequencing

WHO World Health Organization

XLD Xylose lysine deoxycholate agar

NF-kB1 Nuclear factor kappa B subunit 1

NF-kB2 Nuclear factor kappa B subunit 2

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Chapter 1. **Introduction.**

The neonatal period, which is the first four weeks of the new-borns' life, is considered the most vulnerable stage for neonates survival, and very critical period with regard to infection. However, despite this understanding, deaths among this group is still about 2.7 million per annum worldwide of which about two million die in the first week 75% of total. This number accounts for approximately 45% of deaths in children aged below five years (UNICEF 2017).

According to UNICEF (2017) and United Nations Sustainable Development, (2017) , the annual death in children under-five has declined by about a half in 2015 compared with 1990, from 90 to 43 per 1000 live births, and from 12.7 million in 1990 to 5.9 million (47%) in 2015 . In neonates, the number of deaths has dropped from 5.1 to 2.7 million, and from 36 to 19 per 1000 live births in same period. However, although the neonatal death rate, mainly in the first seven days after birth has not significantly decreased, the mortality rate in children and new-borns has markedly reduced over the last two decades. (UNICEF, 2015; United Nations Sustainable Development, 2017).

Mortality rate in neonates before their fifth birthday is about 1.7 times higher in rural regions than urban areas, and survival among neonates with educated mothers is almost three times higher than those with non-educated mothers (United Nations Sustainable Development. 2017). This suggests comprehensive efforts are required to provide the essential services in the non-developed countries where the education is limited to make mothers aware of the risk of pathogenic microorganisms on their infants, and accelerate the speed of progress to terminate preventable child deaths.

Despite the efforts of World Health Organisation and other United nation's bodies concerning with reducing child mortality including Millennium Development Goal four (MDG4), about 223 million children under five years died in the period between 1990 and 2013 worldwide (UNICEF, 2013). The Millennium Development Goals (MDGs) of United Nations (UN) represent the most comprehensive commitment in history to addressing poverty and health illnesses in the world. According to the UNICEF report

2014, the Millennium Development Goal 4 (MDG4) aims to reduce the total deaths of children under five to about 30 per 1000 within 2015, this goal has not been achieved as the neonatal mortality rate among children under five is still about 43 per 1000 (UNICEF 2015).

The Child Health Epidemiology Reference Group (CHERG) neonatal group categorized the neonatal death causes into six main reasons: prematurity, perinatal asphyxia, acute infection, diarrheal diseases, congenital deformation and other causes (Figure 1-1). This data based on information obtained from 130 countries which could be divided into two groups, 79 countries with high mortality rate and 51 with low mortality rates (Liu *et al,* 2015; World Health Organization, 2017).

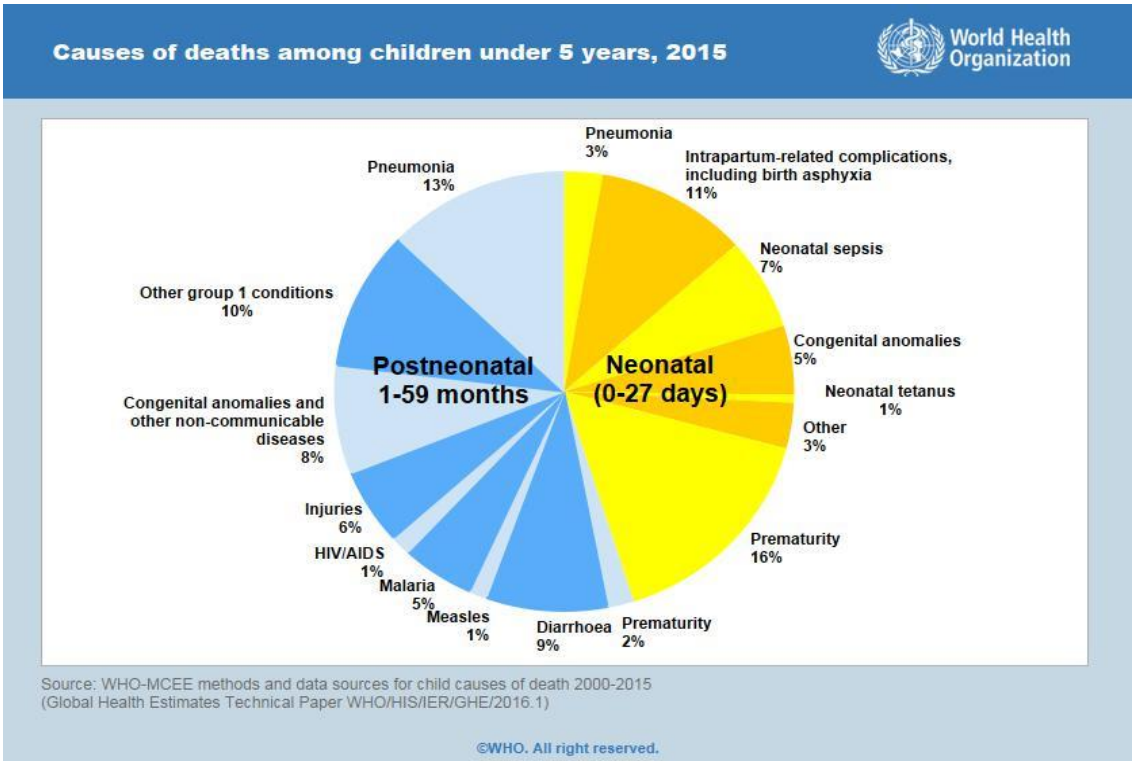


Figure 1-1 Causes of neonatal and children under five years deaths according to WHO 2015 Source: http://www.who.int/gho/child_health/mortality/causes/en/

Recent reports in the UK, indicated that the mortality rate has increased in 2015 to 3.7 deaths per 1,000 births from 3.6 in 2014, and highlighted many risk factors that contribute to increased neonatal mortality including immaturity, birthweight, mother's age at birth of child, and the parents' socio-economic status. (Black *et al,* 2010; Liu *et al,* 2012; Patel, 2017)

In 2015, 16,000 of children under five continue to die every day, mostly from protectable causes (Un.org, 2017). There must remain a strong focus on the survival of children in this vulnerable period, and new sustainable development agenda is vitally required to reduce neonatal mortality especially in the first week of life mainly in countries of high mortality rate.

1.1 Infectious diseases in neonates

The premature infant is highly susceptible to infection and one route is through early-contaminated nutrition. The main clinical risk factors in neonatal infection include the presence of medical devices such as nasogastric enteral feeding tubes, immaturity of their epithelial and mucosal barriers, intravascular catheters, and the immaturity of their immune system (Ramasethu, 2017). The gastrointestinal tract of the neonate may be sterile at birth, yet it is rapidly colonised by bacteria afterwards (Kaufman and Fairchild, 2004; Liu *et al,* 2015).

Often premature infants are fed by a nasogastric enteral feeding tube, with food types ranging from expressed breast milk, to reconstituted powdered infant formulas (PIFs) (Hunter *et al,* 2008). None of these food types can be considered sterile (Drudy *et al,* 2006). These tubes provide a location where bacteria can attach, multiply and produce multispecies biofilms that then slough off clumps of bacteria each time fresh feed is pumped through the tube (Kim *et al,* 2006 & Hurrell *et al,* 2009a and Hurrell *et al,* 2009b). Such clumps are likely to be resistant to such pressures as low pH of neonatal stomach, and so survive in higher numbers.

The care environment is a potential major source of infection to the infants. Previous studies reported that healthcare practice in neonatal intensive care units such as antibiotic exposure play an important role in the nosocomial infections and intestinal sepsis occurrence in premature neonates. The work of Hurrell *et al,* (2009a) and (2009b) at Nottingham Trent University (NTU) has demonstrated that nasogastric feeding tubes can be rapidly colonised with a variety of potentially pathogenic organisms.

Microbial infections are estimated to be responsible for up to 50% of neonatal deaths, with bacteria thought to be the major cause of worldwide deaths in neonates and

accounting for 26% of the deaths (Talbert *et al.,* 2010). However, this average may account for a greater proportion in countries with the highest neonatal mortality rates (Seale *et al,* 2009) where the majority of children are born outside of hospitals in rural areas (Bang *et al,* 2001). However, due to the limitation of microbiology laboratory facilities in poorer countries, less data is available about the causative organisms, especially if infection has occurred during a nosocomial infection or following delivery in hospital or at home (Talbert *et al,* 2010).

Serious bacterial infections can lead to pathologies such as meningitis, sepsis, infection in the respiratory system, and neonatal diarrhoea, which all together are the main causes of neonatal deaths (Cortese *et al,* 2016). Death in the early 7 days of new-born's life is highly attributed to cross-infection during the labour process from colonized mothers, whereas deaths during the later neonatal period is most likely due to acquisition of microbes from the environment, child handling and other social behaviours (Camacho-Gonzalez *et al,* 2013; Chan *et al,* 2013). The dominant microorganisms in neonatal infections therefore could differ according to geographical conditions and microbial surveillance in specific locations (Darmstadt *et al,* 2011; Cortese *et al,* 2016).

Neonatal infection in non-developed countries can vary from 3 to 20 times higher than in developed countries. Moreover, infants who were born at hospitals in less developed countries are more vulnerable to infection with environmental Gram-positive bacteria including *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas* spp and *Klebsiella* spp, due to the lack of some basic requirements like sterilized tools and delivery rooms, poor hygiene, lack of education and unqualified nursing team (Talbert *et al,* 2010; Darmstadt *et al,* 2011).

Group B *Streptococcus* (GBS) is the leading cause of early-onset neonatal infection, with 70% was reported on the first day of birth, whereas the majority of rest occurs in 24 to 48 hours, and only 8% occurred after 48 hours from birth. However, late-onset GBS infection was more likely associated with meningitis and bacteraemia (Koenig and Keenan; 2009). Despite the significant decline in the GBS early-onset sepsis (EOS), this group of bacterium continues to be responsible for high mortality and morbidity rates in elderly people (Koenig and Keenan 2009).

1.1.4 Neonatal sepsis.

Sepsis is one of the highest causes of neonatal mortality and morbidity globally, especially among pre-terms and term neonates with low-birth weight (LBW) that less than 1500g (Stoll *et al,* 2002a and 2002b; Persson *et al,* 2007). Although, sepsis immunopathogenicity is very complex, it can be described as a systemic inflammatory response to pathogenic infection. However, if sepsis was confirmed by isolation of the bacteria from blood it can then be termed bacteraemia (Chen *et al,* 2011; Darmstadt *et al,* 2011 and Shah and Padbury, 2014). Early-onset sepsis (EOS) normally occurs in the first 72 hours after birth because of cross infection with pathogens from the neonates mother at birth, whilst the Late-onset sepsis (LOS) results from a contaminated environment and care provider hands at home or in intensive care units (ICU) after 72 hrs from the birth (Sivanandan *et al,* 2011; Hornik *et al,* 2012)

The pathogen responsible for neonatal sepsis can vary according to the time of infection and region, particularly between industrialized and unindustrialized countries (Sivanandan *et al,* 2011). Reports from developed countries indicates that group B streptococci (GBS) is the major important causative agent of sepsis and meningitis mainly EOS, this is different in some developing countries in the Pacific and Asia where *Pseudomonas* species and *Acinetobacter* species are more common (Darmstadt *et al,* 2011 & Shah and Padbury, 2014)

1.1.4.2 Early-onset sepsis (EOS) microorganisms

In early-onset sepsis, Shah and Padbury (2014) stated that the Gram-positive *Streptococcus agalactiae* (GBS) encapsulated bacterium, is still most often responsible for neonatal sepsis in the US. Shah and Padbury (2014) also stated that *Escherichia coli* is the main causative agent of sepsis in preterm neonates and the second main causative agent for in-term born neonates. However, in the study of Li *et al* (2013) at neonatal intensive care unit Shenzhen Maternal & Child Care Hospital (SZMCH), China, they found out that 83.33% of early-onset sepsis cases were due to Gram-positive bacteria and 66.67% due to Coagulase-negative Staphylococci(CoNS) alone, while only 16.67% resulted from Gram-negative bacteria (Li *et al,* 2013). In contrast, the finding of Wu *et al* (2009) in Taiwan, showed that *E. coli* was the second most common pathogen in EOS

after GBS, but was linked with higher mortality rate than GBS (Wu *et al* 2009). Therefore, the key organisms linked to this pathology still seem to be location dependant.

1.1.4.3 Late-onset sepsis (LOS) microrganisms.

As mentioned before, LOS is more commonly associated with infant environmental conditions rather than vertical infection from the mother. Shah and Padbury (2014) state that about 70% of infants with LOS in infants admitted to National Institute of Child Health and Human Development (NICHD) Neonatal Research Network (NRN) centres/US was due to Gram-positive bacteria, and coagulase-negative staphylococci (CoNS) counted for 48% of them. Sivanandan *et al* (2011) reported that there are some differences between industrialized and unindustrialized countries regarding the organisms responsible for LOS. Approximately, 70 percent of all LOS infant cases in developed countries were due to infection with Gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus* spp and GBS, and the vast majority of infections were in the very low birth weight neonates (Sivanandan *et al,* 2011). In contrast, less than 20 percent were caused by Gram-negative bacteria and most of them belonged to family Enterobacteriaceae (Muller-Pebody *et al,* 2010). On the other hand, in non-developed countries, Gram-negative *E. coli* and *Klebsiella* species followed by *S. aureus* are the main causes of EOS, while the most cases of LOS were *Staphylococcus aureus, Streptococcus pneumoniae* and *S. pyogenes* (Stoll *et al,* 2002; Isaacs, 2003).

1.1.5 Neonatal meningitis.

Meningitis is a serious neurological inflammation of the membranes covering the spinal cord and brain (dura, pia and arachnoid), simply it is an inflammation of the meninges (Saberi and Syed, 1999 and Ward *et al,* 2010). The British physician Thomas Willis (1621–1675) and Italian anatomist and pathologist Battista Morgagini (1682–1771) were the first two people who described meningitis as a specific disease. In 1806, at Medfield, US, the first potential epidemic of bacterial meningitis occurred according to recorded data (Saberi and Syed, 1999). There are several possible causes of meningitis both infectious and non-infectious causes. Non-infectious factors such as narcotic drugs, autoimmune disorders, malignancy, while infectious agent include bacteria, viruses and other

microorganisms like parasites and fungi (Ward *et al,* 2010). The most common microorganisms involved in bacterial neonatal meningitis according to data obtained from four different neonatal centers over the world are: enteric Gram-negative bacilli, and Group B *Streptococcus* (GBS), while *Streptococcus pneumoniae* and *Neisseria meningitis* as well as Type b and non-typable strains of *Haemophilus influenzae* more often caused meningitis in older infants, children and adults. (Nizet and Klein, 2011).

In fact, *Escherichia coli*, a Gram-negative bacterium is one of the most common causes of meningitis in neonates after GBS (Alkeskas *et al,* 2015). This strain possesses an iron acquisition system on a 134-kDa plasmid which is one of most important pathogenic virulence factors of this bacterium, and loss of this plasmid results in a decrease in the pathogenicity by up to 100-fold in meningitis strains (Nizet and Klein, 2011). Among *E. coli* species, K1 serotype is the most common pathogen linked to meningitis in neonates and the fatality rate was significantly higher compared with other *E. coli* infectious strains (Logue *et al,* 2012**;** Alkeskas *et al,* 2015).

In some areas like Netherlands, presence of K1 antigen in cerebrospinal fluid (CSF) was associated with high severity of disease (Nimmich *et al,* 1981; Yao, Xie and Kim, 2006). Besides, studies have shown that this pathogen has many other virulence factors such as *ibeA*, a gene encoding for invasion protein and is a one of the key virulence factors responsible for meningitis in human neonates, outer membrane protein A (OmpA), and Cytotoxic necrotizing factor 1 (CNF1) which confer greater capability to penetrate the host blood brain barrier into bloodstream. (Stins *et al,* 2001 & Nizet and Klein, 2011 & Wang and Kim, 2013).

Friedemann (2009) reported that meningitis accounts for about 42% of neonatal *Cronobacter*-related mortality, and 94% of children that survived *Cronobacter* meningitis develop life threating neurological disorders including impaired sight and hearing and developmental obstacles (Drudy *et al,* 2006).

1.1.6 Inflammatory diseases.

Inflammation is a natural response to microbial infection or tissue damage and includes many different mechanisms that organize the host defense systems. This process is

controlled by the endogenous mediator s of the innate immune system called pro-inflammatory cytokines such as interleukin-8 (IL-8), interleukin-6 (IL-6) and tumour necrosis factor (TNF- α). However, inflammation can cause a variety of pathologies such as meningitis which is inflammation of the meninges (Ward *et al,* 2010). These cytokines play a crucial role in the regulation of the systemic infections.

Inflammatory cytokines such as IL-6, IL-8, and α are mostly produced in response to detection of foreign antigenes such as pathogenic microorganisms in order to activate the immune system and triggering immune cells and other defense agents to the site of infection. Other cytokines such as IL-4 and IL-10 are very important anti-inflammatory cytokines that plays a vital role in controlling and regulating the production of pro-inflammatory cytokines (Kühn, *et al,* 1993; Sellon *et al,* 1998; Sabat *et al,* 2010; Ouyang *et al,* 2011). In addition, transforming growth factor-β (TGF- β) is important in regulation of host immune response and cells differentiation and proliferation, and plays an essential role in tissue homeostasis (Malipiero *et al,* 2007).

1.1.6.2 Neonatal necrotizing enterocolitis (NEC)

Necrotizing enterocolitis (NEC) can also be considered as an inflammatory disease and is strongly linked with inappropriate activation of the immune system and exaggerated induction of inflammatory cytokines such as IL-6, IL-8, and TNF-α in response to infection with pathogenic microorganisms, and is associated with high mortality in neonates (Fusunyan and Nanthakumar, 2001; Lin and Stoll, 2006).

Necrotizing enterocolitis is one of the most serious diseases in neonates, and occurs mostly in premature and low-birth weight newborns, it affects up to 10% of premature infants with symptoms of acute intestinal inflammation in the neonatal bowel (Claud, 2009; Neu and Walker 2011 & Chu *et al,* 3013; Shah and Padbury, 2014). NEC has been linked to different possible reasons including prematurity, microbial colonization, hypoxia/altered intestinal blood flow, and enteral feeding (Claud, 2001). The first use of the term necrotizing enterocolitis was in the middle of the last century by the Europeans Schmid and Quaiser who used this term to describe gastrointestinal necrotic injuries in dead infants (Lee, & Pollin, 2003).

Prematurity which is a birth before 37 weeks of gestation as described by World Health Organization (WHO) (UNICEF 2014) is the most associated factor for NEC development (Lee and Pollin 2003). NEC results in more than 2600 deaths in neonates annually in the USA alone (Dimmitt and Moss, 2001). In developed countries, preterm birth (PTB) accounts for about 70% of total deaths, with serious complications in surviving infants including mental disorders and respiratory problems as well as significant economic effects on the national finances (Karen and Fergal 2012). In addition to immaturity, the undeveloped intestinal epithelial cells may play an important role in the risk of increased intestinal damage. An example of the intestinal immaturity is the limitation of secretion of gastric acid that plays an important role in developing and regulationcommensal flora.

NEC is normally started with a non-severe illness and can progress to serious complications that can be fatal. Dr. Martin Bell was the first to propose the original clinical criteria used to stage NEC cases in 1978**,** and since then, the staging of NEC was developed according to the disease understanding. (Gregory *et al,* 2011), as detailed in table 1-1.

Table 1-1. The modified bell staging criteria for necrotizing enterocolitis NEC according to (Gregory *et al,* 2011).

Stage	Classification	Systemic signs	Intestinal signs	Radiologic signs
IA	Suspected NEC	Temperature instability, apnoea, bradycardia, lethargy	Increased pregavage residuals, mild abdominal distention, emesis, guaiac-positive stool	Normal or intestinal dilation, mild ileus
IB	Suspected NEC	Same as above	Bright red blood from rectum	Same as above
IIA	Proven NEC – mildly ill	Same as above	Same as above, plus absent bowel sounds, with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
IIB	Proven NEC – moderately ill	Same as above, plus mild metabolic acidosis, mild thrombocytopenia	Same as above, plus absent bowel sounds, definite abdominal tenderness, with or without abdominal cellulitis or right lower quadrant mass	Same as IIA, plus portal venous gas, with or without ascites
IIIA	Advanced NEC – severely ill, bowel intact	Same as IIB, plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, disseminated intravascular coagulation, and neutropenia	Same as above, plus signs of generalized peritonitis, marked tenderness, and distention of abdomen	Same as IIB, plus definite ascites
IIIB	Advanced NEC – severely ill, bowel perforated	Same as IIIA	Same as IIIA	Same as IIB, plus pneumoperitoneum

NEC: necrotizing enterocolitis, I, II, and III refer to the severity of the disease and indicate the developmental stage according to the Bell staging scheme. I: stage 1, II stage 2, and III stage 3. Source: (Gregory et al, 2011).

However, the mechanisms involved in the pathological process of NEC are still in doubt, and aetiology and pathogenesis of NEC continue to be incompletely understood, and more likely is a multifactorial disease due to prematurity, lack of human milk feeding and/or microbial colonization (Neu and Walker, 2011). Therefore, the treatment and overall mortality and morbidity have not significantly improved over the last 40 years (Chung *et al,* 2001, Neu and Walker 2011 and Chu *et al,* 3013). Histopathological findings suggested that NEC is a development of widespread necrosis, with a possible role of apoptosis causing primary damage of intestinal epithelial cells before the evolution of the disease (Figure 1-2) (Clark *et al* 2005; Tanner *et al,* 2015).

Recent studies have suggested the possible association between susceptibility and severity of NEC and host genetic factors. Pattern recognition receptors (PRR) is the first line that recognize the pathogen-associated molecular patterns (PAMPs), and regulate intestinal inflammatory response, therefor, different studies have investigated the role of PRR genes variation in NEC (Cuna and Sampath, 2017).

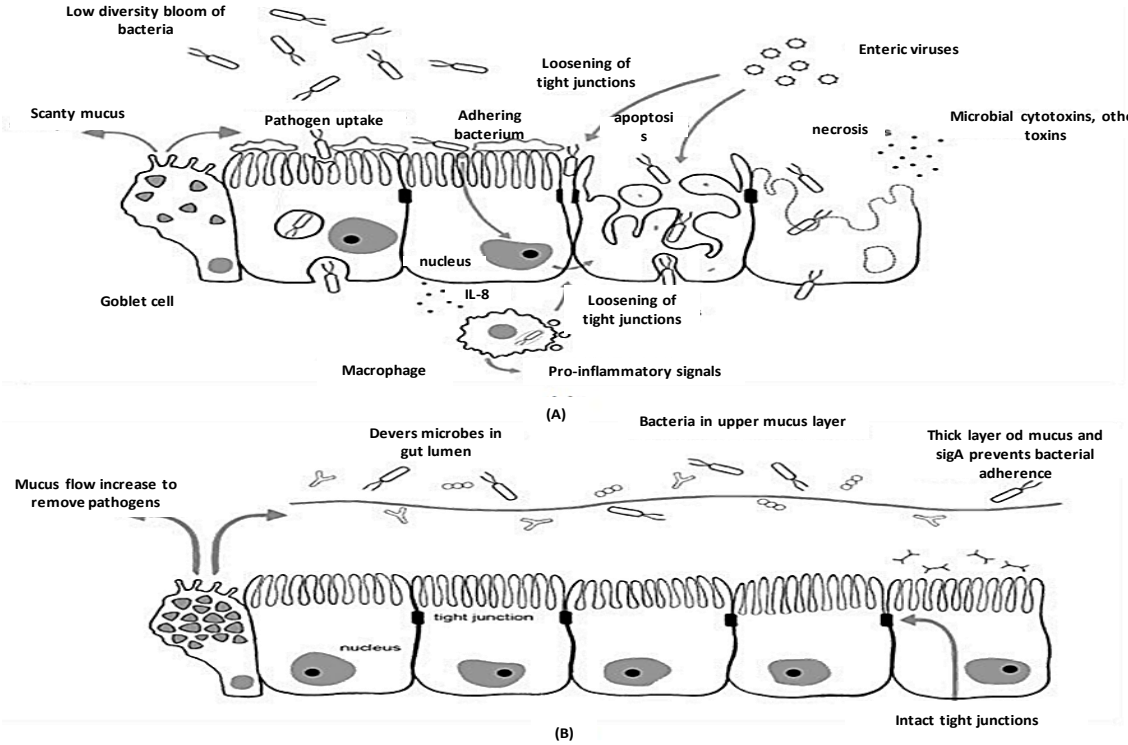


Figure 1-2 ''Progression of events leading to necrotising enterocolitis in the preterm infant.
A bloom of bacteria due to low intestinal motility increases phagocytic uptake. Scanty mucus and reduced antimicrobial factors allow bacteria to adhere to enterocytes, activating NFKB via TRL leading to enterocyte apoptosis, necrosis and loosening of tight junctions. Bacterial translocation may be increased by presence of enteric viruses or microbial and other toxins. b) Intestinal epithelium of the healthy, term neonate. Diversity of enteric microbiota and normal gut motility prevent a bloom of one type of bacteria. Thick mucus, secretory Immunoglobulin A (sIgA) and other antimicrobial factors inhibit bacterial adherence''. Source: (Brooks *et al,* 2013).

Among the PRRs thought to be involved in development of the NEC are Toll-like receptors (TLRs) and Nucleotide Oligomerisation Domain 2 (NOD2) (Le Mandat Schultz *et al,* 2007). Variation of TLR4 expression levels in intestinal cells was thought to play a central role in NEC pathogenesis (Leaphart *et al,* 2007). Zhou *et al,* (2015) reported that the severity of NEC was associated with polymorphism effect on the transcriptional activities of myeloid differentiation-2 (MD2) that is a crucial element of the TLR4 signalling pathway (Kim *et al,* 2013) and essential for binding of bacterial lipopolysaccharides (LPS) to TLR4 (Shibata *et al,* 2011)

Yurdakok (2008) reported low incidence of NEC in low-birth weight infants with interleukin 4 receptor (IL-4ra) allele mutation. This finding supports the previous study by Treszl *et al* (2003) who suggested the role of IL-4Ra and IL-4 in increased Th-2 cell immunity which plays an important role against NEC development. In contrast, Sampath *et al,* (2011) did not find any association between the variation of TLR2, TLR4, TLR5, and TLR9 and interleukin-1 receptor-associated kinase 1 (IRAK1), with the incidence of NEC.

In addition, the unbalanced stimulation of neonatal inflammatory response was also suggested to play an important role in NEC incidence. Franklin *et al,* (2015) reported possible association between the unbalanced IL-6 production and NEC development in Caucasian neonates. Maheshwari *et al,* (2014) found a potential link between NEC severity and different factors including low levels of blood TGF-β and increased IL-8 production compared with controls.

1.2 Bacterial outbreaks in neonatal intensive care units.

Nosocomial outbreaks in neonatal intensive care units (NICUs) have become of more concern in medical research than other types of intensive care units. This is because the improvement in survival of very low birth weight newborns has increased the risk of serious health care-associated infections and subsequently an increase in mortality rate and total cost for the health care system (Stoll *et al,* 2002; Brady 2005; Boghossian *et al,* 2013).

The Gram-negative bacilli Enterobacteriaceae family has been increasingly involved in worldwide neonatal infections, and recently known as the main causes of outbreaks in (NICUs. Enterobacteriaceae members including, *Enterobacter* species *Klebsiella*, *Serratia*,

and *Staphylococcus*, including MRSA, and Extended Spectrum Beta-Lactamases (ESBLs) producers were responsible for most reported outbreaks (Stapleton *et al,* 2016; Ramasethu, 2017).

K. pneumoniae is able to cause urinary tract infections (UTI) and pneumonia in neonates (Hirsch and Tam, 2010). Among Enterobacteriaceae*, Klebsiella* spp. and *Serratia* spp accounts for 23.4% and 13.8% of cases respectively (Gastmeier *et al,* 2007). *E. coli* is involved in a wide range of severe infections with different pathologies and it is the second most common cause of all early-onset infections after Group B *Streptococcus* (GBS) including sepsis and meningitis (Stoll *et al,* 2011).

In addition to previous mentioned bacteria, some *Salmonella* spp. are significantly associated with high morbidity and mortality in neonates (Friedland *et al,* 2003; Adamson *et al,* 2012). However, the common pathologies that are associated with Enterobacteriaceae infections are listed in table 1-2, which adapted from (Fraser and Arnett, 2006; Chaudhry *et al,* 2007; Gastmeier *et al,* 2007; Hammerum and Heuer, 2009; Hirsch and Tam, 2010; Bisi-Johnson *et al,* 2011; Stoll *et al,* 2011; Liu *et al,* 2012).

Table 1-2. The most common clinical species of family Enterobacteriaceae that are known to cause infections in neonates and adults.

Genus	Clinically important species	pathology
Citrobacter	*freundii*	Pneumonia, meningitis, septicaemia, wound and
Enterobacter	*aerogenes* and *cloacae*	Pneumonia, septicaemia, wound and urinary tract
Escherichia	*coli*	Diarrhoea, meningitis, septicaemia and urinary tract
Klebsiella	*oxytoca* and *pneumonia*	Pneumonia, septicaemia and urinary tract infections.
Morganell	*amorganii*	Septicaemia and urinary tract infections
Plesiomonas	*shigelloides*	Diarrhoea and septicaemia.
Providencia	*Rettgeri* and *stuartii*	Urinary tract infections.
Salmonella	*enteritica*	Diarrhoea, typhoid fever, septicaemia, osteomyelitis
Serratia	*marcescens* and *liquefaciens*	Pneumonia, septicaemia, wound and urinary tract
Shigella	*sonnei* and *flexneri*	Diarrhoea.
Yersinia	*Pestis* and *enterocolitica*	Diarrhoea, septicaemia plague and enteritis.
Cronobacter	*sakazakii* and *malonaticus*	Meningitis, sepsis and necrotizing enterocolitis (NEC).

The most common pathogens from Gram-negative Enterobacteriaceae and their pathology. These data were adopted from: (Fraser and Arnett, 2006; Chaudhry *et al,* 2007; Gastmeier *et al,* 2007; Hammerum and Heuer, 2009; Hirsch and Tam, 2010; Bisi-Johnson *et al,* 2011; Stoll *et al,* 2011; Liu *et al,* 2012).

1.3 The genus *Cronobacter*.

The members of genus *Cronobacter* are Gram negative, catalase positive, oxidase negative, methyl red negative, able to reduce nitrate to nitrite, peritrichously motile and facultatively anaerobic rods from the family Enterobacteriaceae with close relation to *Enterobacter* and *Citrobacter* genera (Iversen *et al,* 2008).

1.3.4 *Cronobacter* physiology

In addition to the above characteristics, *Cronobacter* grow over a wide temperature range of 4^{o}C to 44-47^{o}C (Iversen *et al,* 2004). Moreover, members from this genus are well known for their tolerance to desiccation for more than 2 years and rapidly grow on re-constituted powdered infant formula (PIF) (Caubilla-Barron *et al,* 2007). Members of *Cronobacter* are able to attach and colonise the preparation and reconstitution of infant formula equipment used in the manufacture environment or at the newborns preparation equipment including Glass, stainless steel, silicon, polyvinyl chloride, latex and polycarbonate (Iversen *et al,* 2004; Lehner *et al,* 2005). Moreover, most *Cronobacter* isolates are found to produce high amounts of capsules on different agar medium including milk agar plates (Caubilla-Barron *et al,* 2007). This capsule is thought to aid in biofilm formation on different surfaces, which consequently increases its resistance to disinfectants (Beuchat *et al,* 2009). Members of this genus have been isolated from neonatal enteral feeding tubes with with members of other genera (Hurrell *et al,* 2009a, b).

1.3.5 Taxonomic reviews

Cronobacter genus has undergone different taxonomic status during the past 3 decades. Firstly, *Cronobacter* was known as yellow-pigmented *Enterobacter cloacae*, then reclassified as a new species called *Enterobacter sakazakii* based on a genetic study by Farmer *et al,* (1980).

Two decades later, the gene sequences analysis of this bacterium by Iversen and colleagues (2004) using 16s rDNA and *hsp60* showed a close relationship between *E. sakazakii* and *Citrobacter*. Their study distinguished four clusters within *E. sakazakii* each a potential new species (Iversen *et al,* 2004; Iversen *et al,* 2006) which finally resulted in

reclassifying of *E. sakazakii* as a new genus of family *Enterobacteriaceae* called *Cronobacter* (Iversen *et al,* 2007; Iversen *et al,* 2008).

Because of the genetic similarity between members of genus *Cronobacter*, 16S sequence analysis was unable to discriminate between two of the new species (*C. malonaticus* and *C. sakazakii*). Thus, the Multilocus Sequence Typing (MLST) scheme of 7 housekeeping genes described by Baldwin *et al,* (2009) to distinguish between the *Cronobacter* species. These genes are: elongation factor G (*fusA*), ATP synthase b chain (*atpD*), glutamate synthase large subunit (*gltB*), glutaminyl tRNA synthetase (*glnS*), DNA gyrase subunit B (*gyrB*), phosphoenolpyruvate synthase A (*ppsA*) and translation initiation factor IF-2 (*infB*) were used to discriminate the species within *Cronobacter* genus (Baldwin *et al,* 2009; http://pubmlst.org/*cronobacter/*). The scheme was also used to describe the *Cronobacter* new species *C. universalis* and *C. condimenti* (Joseph *et al,* 2012a). According to the recent classification, this genus now consists of 7 species; *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. condimenti C. universalis* and *C. dublinensis* (Iversen *et al,* 2008, Joseph *et al,* 2012).

Published data indicates that some species such as *C. sakazakii* and *C. malonaticus* are more likely related to clinical sources, while others are most likely less frequently associated with clinical hazards (Holy and Forsythe, 2014 and Jackson *et al* 2014). In addition, using the MLST scheme allowed identification of specific associations of some *Cronobacter* Sequence Types (STs), for instance; isolates of *C. sakazakii* ST1 members were associated with infant formula and clinical sources, the ST8 strains are mostly linked to clinical sources whereas ST4 and Clonal Complex 4 (CC4) were dominantly linked neonatal meningitis over the last five decades, while *C. malonaticus* ST7 was linked to adult infections (Joseph *et al,* 2012a; Hariri *et al,* 2013). This scheme is available at: (http://pubmlst.org/*cronobacter/*) (Forsythe *et al,* 2014).

1.3.6 *Cronobacter* ubiquity

Cronobacter has been isolated from different sources such as the outside environment, animals and human (Holy & Forsythe, 2014), and food including herbs, spices, salads, cereals, ready to eat food, vegetables, meat and confectionary (Iversen & Forsythe, 2004; Friedemann 2007; Baumgartner *et al,* 2009). *Cronobacter* triggered researchers attention

around the world due to the presence in powdered infant formula (PIF) that is used to feed neonates and infants as a substitute for breast milk (Iversen & Forsythe, 2004). This however, is unsurprising as PIF product is not sterile and may contain different bacterial isolates of Enterobacteriaceae family (Kucerova *et al,* 2011).

1.3.7 Infections caused by *Cronobacter*

Different *Cronobacter* species have been implicated in serious pathologies, yet *C. condementi* has not been linked to any infection (Cruz-Cordova *et al,* 2012). These pathologies include meningitis, necrotising enterocolitis (NEC), bacteraemia (Healy *et al,* 2010), and sepsis which is mostly associated with neonatal infections with *C. sakazakii*, *C. turicensis* and *C. malonaticus* (Joseph *et al,* 2012a, b).

Cronobacter genus is considered as an opportunistic pathogen largely associated with serious infections in newborns generally neonates (Bar-Oz *et al,* 2001; Mullane *et al,* 2008), with mortality rate up to 80% (Bowen & Braden, 2006; Friedemann, 2007) and with reported infections cases in older infants (Bowen & Braden, 2006). *Cronobacter* species also been implicated with adult infections particularly in immunocompromised individuals and elderly with reported pathologies including sepsis, bacteraemia, diarrhoea, wound infections, and urinary tract infections (Caubilla-Barron *et al,* 2007; Healy *et al,* 2010; Jaradat *et al,* 2014; Cdc.gov, 2017).

With annual global incidence of *Cronobacter* infection of 8.7 per 100, 000 (FAO/WHO, 2006) in neonates and children weighted 2.5 kg or less at birth, there is a justifiable concern about *Cronobacter* infections, as the majority of neonates that recovered from central nervous system (CNS) infections such as meningitis are likely susceptible to develop lifelong chronic mental disabilities (Lai, 2001).

1.3.8 Outbreaks of *Cronobacter*

The main route of *Cronobacter* entry into neonate's body is thought to be through the gastro intestinal tract (GIT) where they may induce NEC (Liu *et al,* 2012). Susceptibility associated with several factors such as a preterm birth neonates, immaturity of neonatal immune system, hypoxia, and consumption of infant formula feed instead of breast milk

that contain protective components and increased bacterial colonisation (Grishin *et al,* 2013).

Cronobacter have been involved in many outbreaks in different parts of the world. The earliest reported data for this species was by Urmenyi and Franklin (1961) in the description of a microbe that caused neonatal meningitis and was described as a yellow pigmented *Enterobacter cloacae*. One of the earlier *Cronobacter* outbreaks was recorded in Netherlands by Muytjens *et al,* (1983) when eight infants developed neonatal meningitis in four different health care units, and six of them died. Five cases were reported in one hospital while the others were from three other hospitals. In this outbreak *Cronobacter* was isolated from prepared infant formula, powdered formula tin and other tools used in preparing formula for infants.

In an outbreak in France 1994, 3 babies died from *Cronobacter* spp infection, two from NEC and one due to meningitis. *Cronobacter* spp. isolates matched the PFGE type of a *Cronobacter* isolated from prepared formula, but did not match the isolates from unopened PIF. In addition, a further 13 neonates showed the presence of the same clonally identical *Cronobacter* isolate, although they experienced symptoms ranging from sepsis to being entirely asymptomatic (Caubilla-Barron *et al,* 2007). However, the virulence of *Cronobacter* could be host dependent, and may remain asymptomatic (Hamby *et al,* 2011; Cetinkaya *et al,* 2013), which was further noted in French outbreak.

In Belgium 2001, *Cronobacter sakazakii* was isolated from 6 neonates out of 12 who developed NEC from which two of them died. *C. sakazakii* was also isolated from prepared formula and unopened cans and after the use of contaminated formula was stopped no more NEC were recorded. This was the first association between *Cronobacter* and NEC.

In a Tennessee hospital, Himelright *et al* (2002) reported that after an infant died of meningitic complications, cultures from eight neonates were positive for *Cronobacter* spp from a total of 49 examined, two of them had respiratory complications. This outbreak was linked with milk formula. Thus, an announcement was made by the manufacturing company to recall the product. Nevertheless, in this outbreak the formula fed to infants was not manufactured by the company for neonatal consumption, by which further questions are raised about the infants' care in those hospitals (Himelright *et al,* 2002).

US 2011, Centers for Disease Control and Prevention (CDC) reported cases of *Cronobacter* spp infections in four different states, when three meningitic infants were recorded and two of them died. *Cronobacter* isolates were sent to Nottingham Trent University for MLST analysis, and results indicated that 14 from a total of 15 strains were *Cronobacter sakazakii* and one *C. malonaticus* (Hariri *et al,* 2011).

In addition to the above outbreaks, *Cronobacter* has been reported to infect the central nervous system (CNS) and cause meningitis in low birth-weight neonates, while bacteraemia and sepsis were more associated with higher birth-weight (Yan *et al,* 2012). Almajed and Forsythe (2015) reported *in vitro* the ability of *Cronobacter* to invade human brain microvascular endothelial cells (HBMEC).

1.3.9 Potential virulence associated factors

While there has been growth in *Cronobacter* research in the last few decades due to the reported infections linked to the organism, the mechanism by which *Cronobacter* cause disease is not yet clearly understood (Jaradat *et al,* 2014). There are virulence associated phenotypic characteristics of *Cronobacter* isolates demonstrated by either epidemiological or *in vitro* studies (Stephan *et al,* 2011). Previous studies demonstrated that neonatal infections were mostly associated with *C. sakazakii, C. turicensis*, and *C. malonaticus* (Healy *et al,* 2010, Kucerova *et al,* 2010). Although, it is not clearly understood how *Cronobacter* cause infection, several potential virulence factors have been determined (Kucerova *et al,* 2010; Stephan *et al,* 2011).

1.3.9.1 Cytotoxic virulence of pathogenic bacteria.

In studies of *Cronobacter* virulence it was found to be able to produce proteolytic enzymes that can contribute damages in the infected tissue in mice (Pagotto *et al,* 2003). In addition to producing proteases, *Cronobacter* isolates can produce enterotoxins active at pH 6 and stable at 90^{o}C for 30 min (Pagotto *et al,* 2003 & Raghav and Aggarwal, 2007). Several enterotoxins (table 1-3) interact with intestinal mucosa and utilize the host membrane activity, including the signal transduction pathways in enterocytes or neuronal, stimulating necrosis and cell death, enzymatic lysing of enterocyte membrane, disruption of enterocyte homeostasis (Popoff, 2011).

Table 1-3 examples of bacterial enterotoxins and their main pathological effects

Site of toxin synthesis	**Toxins**	**Microorganisms**	**Main pathological effects**
Food	Botulinum neurotoxins (intoxination)	*Clostridium botulinum*	Neurological symptoms (flaccid paralysis)
	Staphylococcal enterotoxins	*Staphylococcus aureus*	Nausea, abdominal pain, vomiting
Intestinal lumen subsequent to bacterial growth without bacterial adherence to epithelial cells	Botulinum neurotoxins (toxic infection)	*C. botulinum*	Neurological symptoms (flaccid paralysis)
Gastric or intestinal lumen after bacterial adherence to epithelial cells	Cholera toxin Heat-labile enterotoxin	Vibrio cholerae	Diarrhea, dehydration
	Vero toxin or Shiga-like toxin	*E. coli*	Diarrhea, abdominal pain, fever
	Heat-labile enterotoxin	*E. coli* *Vibrio, Yersinia*	Diarrhea
Intestinal epithelium after bacterial invasion	Shiga toxin	*Shigella dysenteriae*	Abdominal pain, fever, bloody diarrhea
	Listeriolysin	*Listeria monocytogenes*	Systemic symptoms

Source: (Popoff, 2011)

Endotoxins are complex lipopolysaccharides in the cell wall of Gram-negative bacteria such as *Salmonella, E. coli*, and *Shigella spp*. (Cetin *et al,* 2004a; Cetin *et al,* 2004b). LPS is heat stable throughout the preparation of powdered infant formula, and it enhances the *Cronobacter* spp translocation through intestinal epithelial cells to the blood stream (Townsend *et al,* 2007b). Moreover, LPS increases the impairment of enterocyte migration through activation of Ras homolog A (RhoA), a small GTPase responsible for actin cytoskeleton regulation, which disrupts tissue repair and leads to increased bacterial adhesion and invasion (Cetin *et al,* 2004b). Moreover, bacterial LPS can contribute inflammating response in intestinal cells and thought to play an important role in inflammatory bowel disease (IBD) (Ou *et al,* 2009).

Cronobacter outer membrane protein (OMPs) were reported to contribute to invasion into HBMEC *in vitro* (Mohan & Venkitanarayanan, 2006; Singamsetty *et al,* 2008). Although, *ompA* gene were present in all of sequenced *Cronobacter* isolates, due to the

variability of *Cronobacter* pathogenicity, the role of these proteins is not clear (Joseph *et al,* 2012b). *Cronobacter* OMPs are believed to play an important role in bacterial colonisation on the gastrointestinal tract and pathogenesis (Kim *et al,* 2010; Franco *et al,* 2011b). Mittal *et al,* (2009) reported a possible role of *Cronobacter* OMPs in damaging of different tissues in rat model, and causing meningitis compared with OMP deficient strains.

Another potential virulence factor is the type six secretion system (T6SS) that is associated with bacterial invasion of blood brain barrier (BBB) by *E. coli* K1 (Zhou *et al,* 2012), and found to be not present in all *Cronobacter* isolates (Kucerova *et al,* 2010; Joseph *et al,* 2012b). Moreover, plasmid encoded plasminogen activator *cpa,* has proteolytic activity and can cleave the serum complements components-3 (C3), C3a, and C4b and facilitate bacterial invasion and spreading over the host body.

1.3.9.2 Biofilm formation

Biofilm is an important virulence factor that protects bacteria against different environmental stresses including acid and antibiotics (Ravishankar *et al,* 2003; FAO/WHO, 2006; Kim *et al,* 2007). Previous studies indicated that *Cronobacter* can produce biofilms on different material surfaces such as polycarbonate, stainless steel latex and silicon (Iversen *et al,* 2004; Lehner *et al,* 2005). This virulence factor is a real concern in food stuff manufacturing as these biofilms can act as sources for contamination of the food products (Lehner *et al,* 2005; Hartmann *et al,* 2010). Flagella has shown to play essential role of in biofilm formation by *C. sakazakii*, and 2 hypothetical flagellar proteins (ESA_00281-2) were thought to contribute in development of biofilm in *C. sakazakii* (Cruz-Cordova *et al,* 2012). Moreover, polysaccharide capsule is another important factor in biofilm formation, and in *Cronobacter* encoded by capsular polysaccharides genes *wzABCKM* (ESA_01155-01175) Joseph *et al,* 2012b). (Hurrell *et al,* 2009) investigated the biofilm and capsule formation on the different types of neonatal enteral feeding tube by different isolates from family Enterobacteriaceae, and the highest amount of biofilm was produced by *Cronobacter* isolates. However, their results indicated no relationship between capsule production and biofilm formation.

1.3.9.3 Acid resistance

Oral ingestion is the main route of *Cronobacter* colonisation of the human intestine. Thus, to be able to cause infections in neonates, *Cronobacter* strains must survive the very high acidic environment of the stomach. Dancer *et al,* (2009) reported that *Cronobacter* isolates could survive and grow under highly acidic conditions. Edelson-Mammel *et al,* (2005) showed that 83% of *Cronobacter* isolates showed only one log reduction after exposure to pH 3.5 for 5h at 37^{o}C. Similar results were found when *Cronobacter* isolates could grow at pH 4.5. (Johler *et al,* 2009).

1.3.9.4 Antibiotic and desiccation resistance.

Cronobacter isolates showed high susceptibility to wide range of antibiotics. Fei *et al,* (2017) have investigated the susceptibility of 70 *Cronobacter* isoltes to 21 antibiotics and found that all of the isolates were susceptible to most of these antibiotics, except for ampicillin and amoxicillin-clavulanate. The minimum inhibitory concentration (MIC) of cefazolin was similar within the same ST, but varied between different STs. In Korea, Chon *et al,* (2012) found that about 5.6% of *Cronobacter* isolates from desiccated foods were not susceptible to ampicillin. Some *Cronobacter* isolates showed variable β-lactam activity (Caubilla-Barron *et al,* 2007), by which some were PIF isolates (Zhou *et al,* 2011). Resistance of dry and osmotic stresses activities were also reported for *Cronobacter* isolates. In a study by Barron & Forsythe, (2007), *Cronobacter* strain were recovered from PIF after about 2.5 years.

1.4 Bacterial invasion of host cells.

Adherence to host intestinal cells is an initial step to invade and damage intestinal mucosa to facilitate bacterial translocation to bloodstream. Invasive bacterial infection is one of the major causes of mortality and morbidity in children. Attachment or close physical interaction between epithelial cells and invasive bacteria can lead to internalization. Host membrane receptors can be engaged by bacterial ligands to facilitate the pathogen to activate their uptake and entry into host cells (Eick and Pfister, 2004; Tribble and Lamont, 2010). Invasion of intestinal epithelial cells by some pathogenic bacteria such as *Salmonella* prompts an inflammatory response that leads to the production of

antimicrobial peptides and reactive oxygen Species (ROS) by neutrophils. Thus ROS can oxidize the thiosulfate into tetrathionate, which can be used by *Salmonella* as a terminal electron acceptor (TER) to support its growth in anaerobic conditions. In addition to antimicrobil resistance, this pathogen can overcome the commensal bacteria in this inflamed tissues.

One of the mechanisms of adhesion used by pathogenic bacteria is via binding to host cell fibronectin in the extracellular matrix of eukaryotic tissue as shown for *Cronobacter* spp, which reported by Mohan Nair and Venkitanarayanan, (2007). They indicated that Cytochalasin D a microfilaments (MF) depolymerisation agent inhibited *Cronobacter* spp invasion of INT407 cells by about 93% at a concentration of 2 µM/ml (w/v), and a microtubules (MT) depolymerisation agent Nocodazole produced a dose-dependent reduction in bacterial invasion and reduced *Cronobacter* spp invasion 40% at concentration of 20 µM. Colchicine 10 µM and vinblastine 20 µM, the (MT) depolymerization agents reduced invasion by 40% and 72% in *Cronobacter* spp invasion, respectively (Mohan Nair and Venkitanarayanan, 2007).

Monteville *et al,* (2003) also has examined the effect of pre-incubation of Cytochalasin D with INT407 on the bacterial invasion. Three bacteria were used in this study which were: *Campylobacter jejuni, Citrobacter freundii* and *Salmonella enterica*, and their finding suggests that Cytochalasin D resulted in an increased binding of both *C. jejuni* and *S. typhimurium* to INT407 cells in dose dependant manner, and internalized bacteria were decreased according to the concentration of Cytochalasin D. At the highest concentration (2µM) the attachment of *C. jejuni* was increased by to 182% while the bacterial invasion was inhibited by about 37%. Moreover, there was no significant increase in the number of attached *S.* Typhimurium *and C. freundii* while the invasiveness was supressed for approximately 0.2% and 1.2% respectively compared to non-treated cells.

In a study by Loessner (2008), the effect of Cytochalasin D (MF) and colchicine microtubules (MT) inhibitors on bacterial invasion into Caco-2 cell lines was examined. Interestingly, the invasion of *Listeria monocytogenes* and *Salmonella enterica* to Caco-2 cells was considerably inhibited when the cells were pre-treated with Cytochalasin D. In contrast the invasion efficiency of *Cronobacter* spp was significantly increased by about 700%. However, when Caco-2 cells were pre-incubated with colchicine there was a

noticeable inhibition of the invasion of *Cronobacter* spp, while the invasion of *L. monocytogenes* was increased. The authors suggested that the increase of the invasion by *Cronobacter* spp was most likely because of the disruption of tight junctions.

1.5 Host cells as a model of host/pathogen interaction.

1.5.4 Experimental investigation and research.

To establish infection, gastrointestinal pathogens must survive a number of stresses during ingestion. They must breach the acid stress environment of the stomach (Castanie-Cornet *et al,* 1999; De-Jesus *et al,* 2005; Hurrell *et al,* 2009a). After the pathogen has survived the very low pH in stomach, it has to be able to attack and invade the host epithelium to cause disease. The differentiated polarized epithelial cells working as functional and physical barrier, and protects the host from attack by parasites and harmful enteric pathogenic microorganisms. To better understand mechanisms involved in this interaction between polarized intestinal cells and enterovirulent bacteria, many molecular and physiological investigations were designed by the scientists in this field to determine the virulence genes and virulence traits of different pathogenic microorganisms.

The use of human and other eukaryotic cell lines for host-pathogen interaction research is important. Cell lines simplify the complex *in vivo* processes, and may considerably shorten the period of research in contrast to *in vivo* models (Law *et al,* 2013). Use of these cell lines aids in study of the direct interactions between pathogenic bacteria and its host cells, making the use of intestinal epithelial cells lines an essential component of the study of the host-pathogen interaction dynamics (Law *et al,* 2013).

To mimic the function structure of intestinal epithelial barrier of mature enterocytes, different human and other animals' cell line were established. Among the widely used human cell lines are human colon carcinoma (T84) cell line (Clark *et al,* 2005; Oppong *et al,* 2012), human colorectal adenocarcinoma (HT29) cell line (Hsu *et al,* 2014), and human colon colorectal adenocarcinoma epithelial (Caco-2) cells (Chandrapala *et al,* 2014; Almajed and Forsythe, 2016). In addition, human brain microvascular endothelial cells (HBMEC), macrophage cell line (U937) and human microglial cell line are also used for

mimicking other possible pathologies and are available at different type culture collections such as American Type Culture Collection (ATCC), the European Collection of Authenticated Cell Cultures (ECACC) and Innoprot Technologies.

Although, most commonly used human epithelial cells were derived from colonic carcinoma and its genetic control is abnormal, they are extensively investigated in host/pathogen studies as a model of adult derived cells (Sanderson and Walker, 1995; Liévin-Le Moal; Servina, 2013).

Caco-2 cell is one of the most popular *in vitro* used cell lines in investigation of host-pathogen interaction, and exhibits a well-differentiated monolayer giving high similarity to the small-intestinal microvillus (Meunier *et al,* 1995; Buhrke *et al,* 2011), and exhibits spontaneous differentiation for long term culture (Sambuy *et al,* 2005;). However, Caco-2 cells are derived from an adult colon carcinoma (Buhrke *et al,* 2011), whereas there is a limited data available on using non-malignant neonatal cell lines such as H4 cells for examining bacteria responsible for neonatal infections.

The only non-transformed human epithelial cell line being used in the investigation of the interaction between enterovirulent pathogens and host cells is a non-malignant human fetal primary small intestinal cell line (H4 cells). H4 cell line was established by Sanderson and Walker (1995) as a new model of immature non-malignant human neonatal epithelia cells from the whole small intestine of fetuses aged from 20 to 22 weeks gestation by (Sanderson and Walker, 1995).

Later, Sanderson *et al,* (1996) investigated some of the phenotypic characters of this cell line, and concluded that this cell line has the normal chromosome number (46+XY), and expressed villin and cytokeratins as proof of their epithelial cell source. Unlike other carcinoma cells such as Caco-2 or TE84, H4 cells do not form domes after reaching confluence, suggesting lack of sealing by tight junctions (Sanderson *et al,* 1996). H4 cell was then compared with adult Caco2 and HT29-cl19A in regard to their response to stimulation with IL-1β and TNF-α and IL-8 production, and showed IL-8 production significantly higher than both other cell lines. (Claud *et al,* 2003).

However, researchers must consider that results obtained from culturing of cell lines with pathogenic microorganisms needs an additional proof in an *in vivo* model. This is because of lack of the *in vitro* module to the other host cell types, interactions with immune system,

interaction with natural microbiota, and many of the physiological factors to mimic the reality of bacterial infection in vivo. (Law *et al,* 2013).

However, human intestinal specimens could be a more trustable model in this field of research, but they are not readily available to many laboratories compared with cell lines (Vallance *et al,* 2004).

1.5.5 Tissue culture in determining virulence factors of pathogenic bacteria.

Animal and human cell lines have been extensively used in investigations determining the virulence traits of pathogenic microorganisms as well as effects of environmental stresses and cytotoxic effects of chemicals and live organisms. The choice of suitable cell line mostly depends on the question needed to be answered in a particular experiment. Several studies have investigated the adhesion, invasion and survival of different pathogenic bacteria using non-differentiated Caco-2 INT-407 or, T84 cells (Backert and Hofreuter, 2013)

Bacterial invasion begins after the bacteria attach to the host cell surface (Shoaf-Sweeney and Hutkins, 2008). Pathogens such as *E. coli, Shigella, Salmonella* spp *Yersinia* spp *and Streptococcus pneumoniae,* are able to invade and survive within host cells, causing a number of infectious diseases (Reis and Horn, 2010; Terao, 2012)

The small intestine epithelial cell line Caco-2 is the main cell line used in investigating the mechanisms of human pathogen virulence like adhesion and invasion (Grajek and Olejnik, 2004 & Cenciĉ and Langerholc, 2010). Townsend *et al* (2008a) found that isolates of *Enterobacter hormaechei* were able to invade Caco-2 in different levels.

Chandrapala *et al,* (2014) investigated the role of invasion gene (*inv*) and *OmpA* gene in *Cronobacter sakazakii* invasion into different cell lines including Caco-2. Their results indicated that bacterial invasion was largely affected by *Δinv ΔOmpA* deletion mutant compared with the wild type. Townsend *et al,* (2008b) investigated the attachment and invasion of different *Cronobacter* isolates from French outbreak into Caco-2 cells, and found that all of investigated strains were able to attach and invade Caco-2 cells with variable levels.

Different potential virulence factors of *Cronobacter* pathogenicity were investigated using Caco-2 cells. Choi *et al,* (2014) investigated the role of *mcp* gene located on pCSA2 plasmid in bacterial attachment and invasion to Caco-2 cells. The deletion mutation of this gene resulted in significant reduction in both adhesion and invasion (Choi *et al,* 2014). Moreover, isolates from *C. sakazakii* clinical isolates were found to invade and translocate the polarized monolayer of Caco-2 cells (Almajed and Forsythe, 2015).

H4 cell line has been used in a few studies in the last tow decades with limited bacterial isolates, mainly in investigation of the inflammatory response compared with other adult cell lines. This cell showed IL-8 production higher than Caco2 cells in response to stimulation with interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and bacterial lipopolysaccharide (LPS) (Nanthakumar *et al,* 2000 & Claud *et al,* 2003). Claud *et al* (2004) supported this finding but added that the secretion of IL-8 was significantly higher in response to commensal *E. coli* and pathogenic *Salmonella* SL3201. Although, many of recorded outbreaks in neonatal intensive care units occurred after this cell line was established in 1995, there is as yet no published data about the virulence adhesion and invasion of pathogenic bacterial species to this novel cell line.

1.6 Role of Neonatal immune response in disease incidence.

Premature neonates are more susceptible to illnesses compared to older infants, children and adults. This is because the production of neutrophils in neonatal bone marrow is limited, resulting in decreased protection efficiency during an infection period. Furthermore, these neutrophils have some weaknesses in determination of the site of infection and recognising the pathogenic microbes (Koenig and Keenan, 2009).

Toll-like receptors (TLRs) function as the main activators of the innate immune response through identifying pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) of Gram-negative organisms, through pattern recognition receptors (PRR) on host cells. PRR in some cases, recognize agonists produced by human cells and tissues as a result of an exaggerated stimulation of polymorphonuclear leukocyte (PMNs) in affected tissues in human neonates, which is thought to increase the mortality rate among premature babies. Previous findings suggested that there is a

possibility to reduce this overstimulation through suppressing selected TLR (Fusunyan *et al,* 2001 & Thornton *et al,* 2012).

Unregulated expression of the toll-like receptor TLR-4 due to Gram-negative lipopolysaccharide (LPS) may be displayed by the premature intestine, resulting in an excessive pro-inflammatory response through the NF-kB pathway. This expression is downregulated in term new-borns, which is offering more tolerance towards colonisation by commensal bacteria. This inadequate development of TLR-4 response down-regulation to Gram-negative bacteria, may give an explanation of the exaggerated inflammatory response leading to necrotizing enterocolitis (NEC) in pre-term neonates (Cilieborg *et al,* 2011).

Over all, this highlights the importance of having a neonatal cell line as a model for neonatal infection and the need to characterise the H4 cell line in compression with the recent Caco-2 system.

1.7 Study objectives.

Although *Cronobacter* spp. infections are infrequent, the severity and mortality rate among infected neonates are considerably high. Thus, this project aims to evaluate the non-malignant neonatal intestinal epithelial H4 cell line as a new model of human epithelial cells in investigating of the virulence traits of selected isolates from genus *Cronobacter*, and to compare the obtained results with a human colonic carcinoma epithelial (Caco-2) cell line, the most common used cell line in host-pathogen interaction investigations. In addition to *Cronobacter* isolates, *E. coli* K1 NTU ID 939 was chosen as a comparative strain as this serotype the most frequently associated with neonatal meningitis.

Firstly, to gain better knowledge about the selected isolates they were investigated with regard to a wide range of physiological traits including:

- growth rate of the selected strain was investigated in two ways:

In broth over 32 hours to determine the start and end of exponential and stationary growth phase to ensure that bacteria used in studies were in equivalent growth phases, as well as examining growth as a virulence factor

In antibiotic free tissue culture growth medium in order to confirm levels of growth in such medium to allow better understanding on in vitro host cell interaction experiments.

- Bacterial motility that is important for attachment to host cells, induction of biofilm formation, and host cell inflammatory response.
- Biofilm formation on 24-well plates and enteral feeding tubes which will help to understand how bacteria can survive environmental stresses and build up sites of infection.
- Capsule production from different carbon sources including milk agar medium that is based on infant formula to determine the ability of selected isolates to utilize nutrients from the reconstituted formula.
- Ability of bacterial isolates to produce proteolytic and haemolytic enzymes that can destroy blood cells that important virulence for pathogenic bacteria to damage host barriers and enter blood stream where it can translocate to different body organs.

The results of these analyses are presented in Chapter 3.

Secondly, both cell lines were optimised regarding the confluence required time to avoid over-layering or using non-confluent monolayer which both can give false results.

- As limited data is available about H4 cells and previous studies indicated that this cell line is non-immortalized, cell invasion of selected strains to early and late passages were conducted which will help to understand whether there are any changes in this cell line during the serial passaging.

Thirdly, bacterial isolates were incubated with H4 and Caco-2 cells and different parameters were measured including: attachment, Invasion and translocation. Also, the role of the host cytoskeleton in the bacterial invasion to the H4 and Caco-2 was investigated using eukaryotic cytoskeleton inhibitors.

Moreover, cytotoxicity of the selected isolates to H4 cells compared with Caco-2 cells was determined using Trypan blue viability assay and lactate dehydrogenase leakage assay with further examination of highly cytotoxic strain in the ST3 MLST group.

The results of these analyses are presented in Chapter 4.

- **Lastly** induction of host immune system by selected strains with known pathologies.
  - ✓ Investigation via ELISA of the pro-inflammatory cytokine Interleukin -8 (IL-8) production of H4 cells compared with Caco2 cell line to selected isolates.
  - ✓ Investigating the differences in production of IL-8 when bacteria are in contact with the apical side of the polarized layer, mimicking initial adherence and successful penetration of bacteria through this first barrier to the host tissue.
  - ✓ Selected strains were subjected to further investigation for an extra 12 cytokines and growth factors including Il-8, IL-1β, IL-10, IL-12 (p40/p70), IL-2, IL-4, IL-6, Interferon gamma (IFN-γ), tumour necrosis factor alpha (TNFα), epidermal growth factor (EGF) and others, to help to understand the mechanisms behind development of serious pathologies such as NEC. This assay was conducted using 10-plex magnetic beads compatible with Bio-Plex® 200 platform.
- The activation of host immunity by bacterial infection via human Toll-like receptors (TLRs) was investigated. Changes in the gene expression of TLRs1-10 were determined through Real-Time RT-PCR and q-PCR Assays.

The results of these analyses are presented in Chapter 5.

Throughout *Cronobacter* MLST data base was used to investigate the presence and absence of virulence associated genes for correlation to both phenotype and known pathologies. Available at: https://pubmlst.org/*cronobacter*/

Chapter 2. Material and Methods.

2.1 Safety considerations

To ensure safe working at all times COSHH forms were completed and risk assessment of all the materials and protocols were evaluated, recorded and available for view. All laboratory work was carried out according to Health and Safety Code of practice for Microbiology Containment Level 2.

Good Laboratory Practices (GLP) were followed in dealing with chemicals, medium preparation and handling of microbes, Also GLP were carried out with heat autoclaved waste and disposable materials. All laboratory equipment was operated according to the related manual instructions and safety information. Before beginning tissue culture experiments, Hepatitis B vaccination was received. All contaminated material, and other disposables were autoclaved before disposing according to the recommendations in the material safety data sheets

2.2 Bacterial strains

Bacterial isolates were obtained from the Nottingham Trent University culture collection. Forty-six strains from seven species belonging to the genus *Cronobacter* were used including: 35 isolates of *C. sakazakii*, *C. malonaticus* (4 isolates), *C. dublinensis* (n=2), *C. turicensis* (n=2), *C. condiment* (n=1) and *C. muytjensii* (n=1) and *C. universalis* (n=1). In addition to these strains, *E. coli* K1 strain 939 was used in this study as representative of a related known pathology species. *Salmonella* Enteritidis from the National Collection of Type Cultures (NCTC) and *E. coli* K12 were used in all of tissue culture and related experiment as a positive and negative controls respectively (Table 2-1).

These strains were streaked on Tryptic Soy Agar (TSA) from the culture collection and incubated aerobically at 37^{o}C for 16-24 hours in order to check the purity and subsequently stored in 20% (v/v) glycerol Tryptic Soy Broth (TSB) (Fluka, UK). (Fisher Scientific, UK) at a temperature of -80^{o}C.

Table 2-1: Details of the strains that have been used in this study

NTU no	species	ST	Country	source	Year of isolation	Disease	Site of isolation
658	*C. sakazakii*	1	USA	Non-clinical	2001	Non-clinical	Non-infant formula
1	*C. sakazakii*	8	USA	Clinical	1980	Child's Throat isolate	Child's throat
690	*C. sakazakii*	12	France	Clinical	1994	Asymptomatic	Faecal isolate.
691	*C. sakazakii*	4	France	Clinical	1994	ND	Sputum isolate
692	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant faecal isolate
693	*C. sakazakii*	13	France	Clinical	1994	Asymptomatic	Infant faecal isolate
694	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant conjunctivae isolate
695	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant trachea isolate
696	*C. sakazakii*	12	France	Clinical	1994	NECII	Infant faecal isolate
698	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant faecal isolate
699	*C. sakazakii*	12	France	Clinical	1994	ND	Infant throat isolate
700	*C. sakazakii*	12	France	Clinical	1994	Digestive Problem	Infant faecal isolate
701	*C. sakazakii*	4	France	Clinical	1994	Fatal NECIII	Infant, peritoneal fluid
702	*C. sakazakii*	4	France	Clinical	1994	NECI	Infant faecal isolate
703	*C. sakazakii*	12	France	Clinical	1994	NECII	Infant throat isolate
705	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant throat isolate
706	*C. sakazakii*	4	France	Clinical	1994	NECII	Infant faecal isolate
707	*C. sakazakii*	12	France	Clinical	1994	NECII	Skin isolate from infan
708	*C. sakazakii*	4	France	Clinical	1994	Asymptomatic	Infant throat isolate
709	*C. sakazakii*	4	France	Clinical	1994	Septicaemia	Infant trachea isolate
711	*C. sakazakii*	4	France	Clinical	1994	Asymptomatic	Infant faecal isolate

USA: United States of America; **UK**: United Kingdom; **UN**: Unknown. **ST**: sequence type, **NEC**: necrotizing enterocolitis stage **I**: 1, **II**: 2 or **III**: 3. **NA**: not available.

Table 2-1 continues

NTU no	species	ST	country	source	Year of isolation	Disease	Site of isolation
712	*C. sakazakii*	4	France	Non- clinical	1994	Non clinical	Reconstituted formula
713	*C. sakazakii*	13	France	Infant formula	1994	Non clinical	Reconstituted formula
714	*C. sakazakii*	13	France	Infant formula	1994	Non clinical	Reconstituted formula
715	*C. sakazakii*	13	France	Infant formula	1994	Non clinical	Reconstituted formula
716	*C. sakazakii*	14	France	Infant formula	1994	Non clinical	Infant formula
717	*C. sakazakii*	14	France	Infant formula	1994	Non clinical	Infant formula
718	*C. sakazakii*	14	France	Infant formula	1994	Non clinical	Infant formula
730	*C. sakazakii*	4	France	Clinical	1994	NECI	Infant faecal isolate
767	*C. sakazakii*	4	France	Clinical	1994	Fatal infant meningitis	Infant trachea isolate
978*	*C. sakazakii*	3	UK	Clinical	2007	Clinical	Enteral Feeding Tube
984*	*C. sakazakii*	3	UK	Clinical	2007	clinical	Enteral Feeding Tube
513*	*C. sakazakii*	8	Czech Republic	Clinical	1983	Clinical	NA
1249	*C. sakazakii*	31	UK	Clinical	2010	Fatal infant meningitis	Cerebrospinal fluid (CSF)
563*	*C. sakazakii*	41	USA	Clinical	1975	Foot wound	Foot wound isolate
1557*	*C. sakazakii*	23	Unknown	Clinical	1979	Bronchial secretion	9 year old girl
1546*	*C. malonaticus*	84	Czech Republic	Clinical	NA	NA	Bed swab
1558	*C. malonaticus*	7	Czech Republic	Clinical	NA	NA	Faecal isolate.
1569	*C. malonaticus*	307	USA	Clinical	2011	Fatal infant meningitis	Blood isolate
681	*C. malonaticus*	7	USA	Clinical		NA	Breast abscess isolate

USA: United States of America; **UK**: United Kingdom; **UN**: Unknown. **ST**: sequence type, **NEC**: necrotizing enterocolitis stage **I**: 1, **II**: 2 or **III**: 3; **NA**: not available. Asterisks *: whole genome sequence performed as a part of this project.

Table 2-1 continues.

NTU no	species	ST	country	source	Year of isolation	Disease	Site of infection
581	*C. universalis*	54	UK	Water	1956	Non clinical	Freshwater
564	*C. turicensis*	5	USA	Clinical	1970	Neonatal Meningitis	Blood isolate
1211	*C. turicensis*	19	Switzerland	Clinical	2005	Fatal infant infection	Blood isolate
1330	*C. condimenti*	98	Slovakia	Food	2010	Non clinical	Food
1561	*C. muytjensii*	81	USA	Unknown	NA	Non clinical	Unknown
1556	*C. dublinensis*	88	USA	Clinical	1979	Abscess	base of spine
1210	*C. dublinensis*	106	Ireland	Environment	2004	Non clinical	Milk powder manufacturing plant.
939	*E. coli* K1	95	UK	Clinical	NA	Clinical	Enteral Feeding Tube
358	*Salmonella* Enteritidis Positive control in invasion experiments						
1230	E. coli K12 Negative control in invasion experiments						
NCTC 9994	*Streptococcus pyogenes* NCTC 9994 Positive control for β-haemolysis						
NCIMB 6571	*Staphylococcus aureus* NCIMB 6571 Positive control for α-haemolysis						

USA: United States of America; **UK**: United Kingdom; **UN**: Unknown. **ST**: sequence type, **NEC**: necrotizing enterocolitis stage **I**: 1, **II**: 2 or **III**: 3 and **K*:*** capsule type; **NA**: not available.

2.3 Bacterial long term storing solution

Storing solution was prepared by adding 200µl of 80% glycerol to 800 µl of TSB in a 1.5 ml Eppendorf tube. A pure culture strain was collected and mixed with this solution and stored at -80^{o}c until required.

2.4 Overnight cultures of bacteria

In preparing liquid cultures for experimentation a standard method was adopted. Frozen cultures were streaked onto Tryptone Soya Agar TSA plates and incubated for 18-24h at 37^{o}C, then1-2 colony was inoculated into 5 ml of Tryptone soya broth (TSB) which was previously incubated in shaker at 200rpm for 18-24h at 37^{o}C before use in numerous experimental procedures to avoid any contamination.

2.5 Sterilization

All medium, solutions and buffers, were sterilised by autoclaving at 121°C for 15 min and/or by filtration through a 0.2-µm pore size syringe filter (Fisher Scientific, UK). All equipment was disinfected with 70% v/v alcohol prior to use.

2.6 Preparation of Buffers and reagents

Buffers and reagents were prepared with either distilled water or deionized double distilled and sterilised water as follows:

2.6.1 Phosphate buffered saline

Phosphate buffered saline tablets (PBS) for bacterial serial dilutions was obtained from Fisher Scientific UK Ltd (12821680) and prepared as described by company instructions. One tablet was dissolved in 100 ml of distilled water then autoclaved at 121°C for 15 min. After autoclaving bottles of PBS were stored at approximately 21^{o}C and used as a required. For tissue culture Dulbecco's Phosphate-Buffered Salines (DPBS) were used (Thermo Fosher, 14080089).

2.6.2 Washing buffer.

Wash buffer was prepared to be used in washing steps during the human Interleukin 8 (IL-8) ELISA assay. 50µl of Tween-20 was added to 100ml PBS described above and sterilized in autoclave.

2.6.3 Ethylenediamine tetra-acetic acid, sodium hydroxide EDTA 0.5 M

The 0.5 M EDTA was prepared by dissolving 186.1 g of EDTA in 700 ml of distilled water. The pH was adjusted to 8.0 by adding approximately 20 g of sodium hydroxide (NaOH) from Sigma Aldrich, UK (S8045) and mixed well to dissolve. The volume then was made up to 1 L and autoclaved at 121°C for 15 min and stored at approximately 21^{o}C.

2.6.4 Hydrochloric acid (HCl)

Hydrochloric acid 10 M was used to control the pH of various solutions that used in this study, the stock solution was filter sterilized using 0.22 µM filters.

2.6.5 Sulphuric acid (H_2SO_4)

Sulphuric acid was prepared to 1M concentration from the stock 18M to be used as stop solution in interleukin 8 (IL-8) assay throughout inactivation the horseradish peroxidase (HRP) enzyme due to changes in the pH.

2.6.6 Preparation of 1 molar solution of sodium hydroxide

Four grams of NaOH (sodium hydroxide) were dissolved in 100 ml of distilled water to obtain a 1M stock solution. This was then filter sterilized using 0.2µm syringe filter and stored at 25-28^{o}C. Working solution of 0.1M NaOH was prepared by diluting 1 ml of 1.0 M NaOH solution with 9ml of distilled filter sterilized water and storage at 21^{o}C.

2.6.7 Triton X-100 preparation.

One millilitre of Triton X-100, Fisher Scientific Fluka, Canada (BPE151-100) was added to 99 ml of distilled water(dH_2O) to prepare 1% (v/v) Triton X lysing solution, and autoclaved at 121°C for 15 min. This solution was stored at 21^{o}C and used as required.

2.6.8 Chloramphenicol 20µg/ml.

To prepare a stock solution at a concentration of 34mg/ml, 0.34g of the chloramphenicol powder was dissolved in 10 mil of 100% ethanol. This stock was stored at 2-8^{o}C. Working solution of 20µg/ml Chloramphenicol antibiotic (5.88µl to final volume of 10 ml and stored at -20^{o}C). This was used to inhibit protein synthesis by investigated bacterial isolates and prepared as needed.

2.7 Culture medium preparation.

2.7.1 Tryptic Soy Broth (TSB)

For liquid medium, 15 g of Tryptic Soy Broth (Fluka, UK) base were added to 500 ml of distilled water. The re-suspended solution was then autoclaved at 121^{o}C for 15 minutes. The broth was then stored at 21^{o}C until required.

2.7.2 Tryptic Soy Agar (TSA)

For solid medium, 40 g of Tryptic Soy Agar (Oxoid, UK) base were added to 1 L of distilled water as described by the manufacturer's instructions. This prepared solution was autoclaved at 121^{o}C for 15 minutes. Medium was allowed to cool down to a temperature of 50^{o}C. After the medium had cooled, about 15-20 ml was poured in each new sterile petri dish. Plates were then allowed to solidify and stored in order to be used within 3-4 weeks. Before using the plates, the purity was checked by incubating them for about 24 hours at 37^{o}C.

2.7.3 Xylose lysine Deoxycholate agar (XLD) medium.

According to manufacturer's instructions, 53 grams of Xylose lysine deoxycholate agar medium were added to 1000 ml distilled water. Medium was then boiled (Not autoclaved) using a magnetic stirrer hotplate to avoid burning of the medium. Prepared medium was moved to a water bath at temperature of 50^{o}C for about 20 - 30 min. 15 - 20 ml of medium transferred to each sterile petri dish, after medium had solidified plate were stored in fridge at 4-8^{o}C.

2.7.4 Violet Red Bile Glucose Agar (VRBGA) medium.

One litre of medium was prepared by suspending 38.5 grams of Violet Red Bile Glucose Agar in 1000 ml of distilled water according to the manufacturer's instructions. The suspension was then heated until the medium boiled (not autoclaved) using a magnetic stirrer hotplate to avoid burning of the medium. Boiled medium was moved to a water

bath at a temperature of 50^{o}C for about 20 - 30 min. Approximately 15-20 ml of medium were transferred to each sterile petri dish. After the medium had solidified plates were stored in fridge at 4-8^{o}C until used as described in methodology (Section 2.6.5.1).

2.7.5 MacConkey Agar.

Fifty-two grams of medium powder were suspended in 1 litre of distilled water and boiled to dissolve medium, then sterilised by autoclaving at 121°C for 15 minutes. Medium was then moved to a water bath at a temperature of 50^{o}C for about 20 - 30 min and 15-20 ml medium were poured to each sterile petri dish. After the medium had solidified plates were stored in fridge and used as required.

2.7.6 Violet Red Bile Lactose Agar medium (VRBL) PHENOTYPING

Violet Red Bile Lactose medium (VRBL) was prepared by suspending 38.5 grams in one litre of distilled water. This suspension was mixed and boiled using Magnetic Stirrer Hotplate. Medium was then cooled to about 50°C and 15-20 ml poured into sterile petri dishes. Plates after they had completely solidified, were stored at 21^{o}C and used as required.

2.7.7 Druggan-Forsythe-Iversen (DFI) medium.

The chromogenic medium Druggan-Forsythe-Iversen medium (DFI) agar base (HAL013, Lab M Limited; UK) was used to discriminate *Cronobacter* spp. from other Enterobacteriaceae family members. A 21.5 DFI grams were used to prepare five hundred millilitres of this medium, and autoclaved at 121°C for 15 minutes. After autoclaving, medium left to cool to approximately 50°C and poured into sterile petri dish 15-20 ml each. Plates after completely polymerised, were stored at 4-8^{o}C and used as required

2.7.8 Congo red medium

LBA- Congo red medium was used to investigate phenotypic characteristics of the selected bacterial isolates. Medium was prepared by suspending 10g tryptone, 5g yeast

extract and 15 grams of technical agar (LP0013 Agar No 3; Oxoid Thermo Fisher; UK) in 990 ml of distilled water, and dissolved by hand mixing. Medium was autoclaved at 121ºC for 15 minutes and allowed to cool to approximately 50°C, and then 10 ml of distilled water containing Congo red dye (4 mg/ml) and 20 mg/ml of Coomassie Brilliant Blue R pure (Scientific Laboratory Supplies, B7920-50G) were added to this medium. Medium gently mixed and 15-20 ml of medium was dispensed in each sterile Petri dish. Plates allowed to cool before storing and used as needed.

2.7.9 Milk agar medium.

To prepare one litre of milk agar medium without any source of carbon except the milk, 15 grams of Agar Technical Agar No. 3 (Oxoid, OXLP0013B) powder, were added to 800 ml of distilled water and autoclaved at 121^{o}Cfor 15 minutes. Autoclaved medium was then transferred to a water bath at 50^{o}C for 20 – 30 minutes, and after the medium cooled to about 50^{o}C, 200 ml of Pre-warmed milk (both Cow & Gate and SMA 1 First infant milk) were added to final volume of 1000 ml of each agar based medium and gently agitated to avoid making bubbles. Approximately 20ml molten medium was poured into each sterile petri dish and left to set. Plates were then stored at 4^{o}C and used as required.

2.7.10 Triphenyl- Tetrazolium Chloride (TTCS) motility medium.

The medium for motility assay was prepared by adding 4 grams of t Agar Technical Agar No. 3 (OXLP0013B-Oxoid, UK), and 20 grams of Luria-Bertani broth (LB) (CM0129-Oxoid Thermo Fisher. UK) to one litre of distilled water. Then 5 millilitres of Triphenyl-tetrazolium chloride, solution (TTCS) were added (0.5 ml/100 ml medium) to visualize the bacterial motility as described by Kelly Fulton (1953) to show where growth has occurred. The prepared solution was then autoclaved at 121^{o}C for 15 minutes and allowed to cool to about 50^{o}C, and approximately 15-20 ml of medium was then poured into sterile Petri dishes. Plates were left at 21^{o}C for about 48 hours to solidify before the experiment.

2.7.11 Blood Agar medium.

The ability of *Cronobacter* strains to lyse blood cells was investigated. Tryptic soy agar (20g) was dissolved in 500ml of distilled water according to the manufacturer's instructions and autoclaved at 121°C for 15 minutes. After cooling to 45-50°C, a 5% (v/v) suspension of sheep or horse blood was created by adding 25ml of sterile defibrinated sheep blood (SR0051-Oxoid, UK) or horse blood (SR0050-Oxoid, UK), and gently mixed and subsequently 15-20 ml dispensed into sterile Petri dishes.

2.8 Methodology.

2.8.4 Physiological characterisation of bacterial isolates.

2.8.4.1 Adjustment of bacterial number to the desired concentration.

The bacterial suspension densities used in this project were adjusted to optical density (OD) OD_{600} of 0.05 in all experiment based on bacteria from liquid culture. From -80^{o}C bacterial strains were streaked on agar plates and incubated for 16-24h. Then 1-2 similar colonies were inoculated into 5 ml of TSB medium for 18-24h. 0.1 ml from the bacterial culture were diluted in 0.9ml of appropriate medium and then OD_{600} obtained using plate reader.

OD_{600} of 0.05 was calculated as following: The obtained (OD X 10) – blank = real OD. Then the volume required from the broth culture was calculated using the following formula.

$$\text{volume needed} = \frac{\text{desired volume}}{(\text{real OD}/\text{required OD}) - 1} = \text{amount of bacterial suspension required for the desired volume.}$$

2.8.4.2 Bacterial growth assay.

2.8.4.2.1 Growth caurves in tryptone soya broth medium (TSB).

In order to determine the growth curve, bacterial isolates from -80°C freezer were streaked on TSA plates, and incubated for 16-24 hours at 37°C. One colony was transferred to approximately 5 ml of TSB and incubated in shaking incubator (200rpm) for about 18 hours at 37°C. Then bacteria were adjusted in TSB to optical density of OD_{600} 0.05 and transferred to 24-well plates one ml/well in three replicates. First plate was measured with plate reader at wavelength of 600nm as time zero. The measurement was repeated at the following time points: 3, 6, 9, 21, 24, 27, 30, 32 hours. Bacterial growth curve was performed using Microsoft Excel 2013.

2.8.4.2.2 Growth caurves in tissue culture medium assay

The growth of bacterial strains were also investigated in tissue culture medium. Strains from -80°C were streaked on a TSA plates and incubated for 16-24 at 37°C. One colony was transferred to approximately 5 ml of TSB and incubated in shaking incubator (200rpm) for about 18 hours at 37°C. In tissue culture infection medium (no antibiotic added) , all strains were adjusted to optical density of OD_{600} 0.05 and transferred to 24-well plate one ml/well in three replicates. Plates were then incubated in tissue culture incubator at 37°C and 5% CO_2. Bacterial growth was determined by an increase of the OD_{600} over 3h, simulating a typical tissue culture experiments.

2.8.4.3 Acid sensitivity assay.

The selected strains were investigated in regard to their sensitivity to low pH conditions. Strain from -80°C stock were streaked on TSA plates and incubated 16-24h, then approximately 5 ml of TSB were inoculated with bacteria and incubated overnight in shaking incubator (200rpm). Bacteria then adjusted to OD_{600} 0.05 in TSB pH 7.2 and pH 2.02 ± 0.02 and incubated for two hours at 37°C. Bacteria were then seriality diluted in TSB in 96-well plates up to 10^{-7} and incubated 16-24h at 37°C. Results were calculated as log of the initial and compared with non-treated medium.

2.8.4.4 Bacterial sensitivity to Triton X-100

Bacterial strains were investigated in regard to their sensitivity to Triton X, briefly, 50µl of 18 hours TSB culture were added to 950 µl 1% (v/v) Triton X and incubated for one hour at 37°C. After this period 200 µl of bacterial suspension was transferred into 96-well plates and serially diluted in TSB up to 10^{-7} and then incubated for 16-24h at 37°C. For controls, bacteria were incubated with PBS instead 1% (v/v) Triton and results compared.

2.8.4.5 Sensitivity of bacterial isolates to gentamicin

Invasion assays are also known as a gentamicin protection assays since gentamicin antibiotic can be used to kill extracellular cells and those who are attached to host cells. Thus, the sensitivity of selected bacteria isolates to the concentration of 125mg/ml of this antibiotic was examined by incubation of bacterial strains at OD of 0.05 in the mentioned concentration for one hour. After that bacterial viability was tested using Miles Misra assay, and broth dilution assay.

2.8.4.6 Phenotypical characteristics and colony morphology types bacterial isolates.

2.8.4.6.1 Morphological appearance of bacterial isolates on different medium.

Growth appearance of bacterial strains was examined by using different cultur medium which were TSA, XLD, VRBGA, VRBLA, DFI and MacConkey agar medium. Bacterial isolates were firstly streaked on TSA plates and incubated overnight at 37°C. Then single colony was streaked on appropriate agar medium. The morphology of bacterial growth was verified after overnight incubation at 37°C.

2.8.4.6.2 Congo red morphotypes.

Morphotypical phenotypes of selected bacterial isolates were studied. Bacterial strains were streaked on TSA plates and incubated for 16-24h, and then 1-2 colony inoculated into 5 ml of TSB medium and incubated in shaker at 37°C for 16-24h. Then, 3 µl from this

culture were inoculated onto Congo red agar plates, and incubated 24h at 37°C and or 72h at 28°C. Phenotype for each strain was determined according to the morphological characteristics of the bacterial growth and their ability to bind to Congo red dye at the stated time points. Four different morphologies Congo red dye binding were observed, (**a**): brown, dry, and rough (BDAR); (**b**): red, dry, and rough (RDAR); (**c**): red and smooth (RAS); and (**d**): brown and smooth (BAS).

2.8.4.7 Bacterial motility.

Bacterial strains were streaked on agar plates and incubated for 16-24 hours and then one colony transferred to about 5 ml of TSB, and incubated at 37^{o}C 16-24 hours in shaking incubator at 200 rpm. Three microliters of bacterial suspensions from overnight culture were inoculated in the middle of the pre prepared motility plates and incubated for 24 hours at 37^{o}C. Then the motility was determined as the diameter of growth in mm through the medium.

2.8.4.8 Capsule production assay.

In each case bacteria were grown as described in section 2.4. Bacteria were diluted in PBS to the same OD_{600} and quadrant streaked on various solid medium which are: first infant milk of Cow & Gate and SMA agar medium, XLD and VRBGA. Evaluation of capsule production for the strains was assessed by visual comparison with non-capsulated strains. In each case all investigated strains were graded according to level of capsule produced, with photographic evidence taken of each grade.

2.8.4.8.1 Anthony's Capsule staining method.

Capsule formation by the bacterial isolates was also investigated using the method of Anthony (1931). Briefly, smear was prepared form overnight culture on skimmed milk agar, followed by air drying without heat fixing. Smears were then stained with 1% (w/v) () crystal violet for 2-3 min, and rinsed gently and thoroughly with copper sulphate 20 % (w/v). Slides were then left to dry in the air, and subsequently capsules were

microscopically observed using oil-immersion lenses at 100× magnification. Capsulated bacteria are surround with transparent halo while non- capsulated absorb the stain and looks purplish in colour. (Behare *et al,* 2013)

2.8.4.9 Biofilm formation assay.

Biofilm assay was conducted to determine the ability of selected strains to produce biofilm in milk as the main carbon source according to methods described by (Yoon *et al,* 2011). Two different methods were used:

2.8.4.9.1 Biofilm production in 24 well plates.

Bacterial isolates from 16-24h shaking culture were adjusted to absorbance of OD_{600} 0.05 in breastmilk substitute Cow and Gate(C&G) and simulated milk adapted (SMA) first infant milk ready to feed formula. One ml/well of bacterial suspension was inoculated into each well in the 24 well plate in 3 replicates, and plates were then incubated statically for 24 hours at 37°C. After 24 hours, wells were washed 5 times with sterile distilled water (SDW) and gently inverted on absorbent paper to remove any residual water. Wells were then stained with 200 µl of 1% (v/v) crystal violet solution for 30 minutes. Wells were washed again with SDW until the dye was removed by washing. To measure biofilm production, 200 µl of 70% ethanol was added to the wells and placed for 15min on an orbital shaker at 150 rpm to extract the dye. Finally, 100 µl of this ethanol extract was removed and optical density at 540nm. Non-inoculated standard wells were used as a blank.

2.8.4.9.2 Biofilm production in enteral feeding tubes.

Enteral feeding tubes, Unomedical /Denmark (1.7mm x 40 cm) were cut into small pieces (10mm in length) and placed in a 24 well plate with 4 pieces/well. The bacterial suspension was prepared as described in 2.6.7.1, and gently agitated using orbital shaker (≈200 rpm) for about 30 min to ensure entering of the milk into the tube pieces. All experimental steps and conditions were conducted as described in biofilm production in 24 well plates. These conditions were a modification of the work of Hurrell *et al,* (2009).

2.8.4.10 Blood Haemolysis assay.

A single bacterial colony from an overnight TSA plate was streaked onto a blood agar plate previously prepared (section 2.5.2.11), and incubated at 37°C for approximately 16-24 hours. According to what was observed, strains were categorised as follows: β (beta) haemolysis indicated by clearing around colonies on blood agar, α (alpha) is a greenish colour around the colonies resulted in partial haemolysis and a lack of clearing any other change around colonies categorised as a Gamma haemolysis. *Streptococcus pyogenes* NCTC 9994 and *Staphylococcus aureus* NCIMB 6571 were used as a β and α-haemolysis positive controls respectively.

2.8.4.11 Serum resistance

Bacterial sensitivity to the human serum was determined according to the protocol described by Hughes *et al,* (1982) with minor modifications. Bacteria form the 16-24h broth culture were adjusted to OD_{600} of 0.05 ($1*10^7$/ml) in a frish TSB and incubated for another 2hrs. Following the incubation period, forty microliters of bacterial suspension were mixed with 360 µl of 50% (v/v) of active human serum (Sigma, UK) in new sterile tubes and the mixture were incubated at 37°C at 200 rpm in a shaking incubator. Bacterial isolates were recovered from the serum for the viable counts at four time points (0, 1, 2, and 3 hours) and serially diluted in PBS, followed by incubation on TSA for about 16-24 hours at 37°C using Miles and Misra Method in triplicate. Bacterial survival of each strain was indicated as percent of the inoculum.

2.8.4.12 Genomic studies.

Whole genome sequencing was performed for some strains that available in the *Cronobacter* MLST database. These strains are *C. sakazakii* 513, 563, 1557 and *C. malonaticus* 1546. These strains were sent on TSA plates to Swansea University/ UK, and results were analysed and inserted to the *Cronobacter* MLST available at: https://pubmlst.org/*cronobacter*/ . In addition, *C. sakazakii* strains 978 and 984 were re-sequenced at Nottingham Trent University.

2.8.4.12.1 Genomic DNA Extraction.

Bacterial genomic DNA was extracted using GenElute™ Bacterial Genomic DNA kit (Sigma Aldrcm, UK) according to the manufacturer's instructions. From overnight culture, 1.5 ml was used to extract the DNA for each strain. Then, the purity and concentration of the extracted DNA were evaluated using the Nano-drop 2000 (Thermo Scientific, UK). The minimum accepted concentration was 15ng/µl purity of the investigated samples was 1.8 at 260/280 nm.

2.8.4.13 Outer Membrane Proteins (OMPs) study

2.8.4.13.1 Bacterial strains

OMPs were profiled for 15 bacterial isolates that are: *C. sakazakii* strains 1, 513, 658, 701, 709, 767, 978, 984, 1249 and 1557, *C. malonaticus* strains 681, 1456 and 1569, *C. turicensis* 1211, and *E. coli* K1 strain 939

2.8.4.13.2 Outer membrane proteins (OMP) extraction and profile

The extraction of the outer membrane proteins from bacteria culture was conducted as described by Kim *et al,* (2010a) with some modifications. Bacterial cultures were grown on TSA overnight at 37°C. Up to 10 colonies were picked from the TSA plate, inoculated into 10 ml TSA and incubated in shaking incubator for about 30 minutes until bacterial colonies completely suspended. The suspension then was transferred to a 200 ml of pre-autoclaved TSB which was also previously incubated overnight at 37°C in shaking incubator to check for contamination. Strains were then incubated aerobically overnight at 37°C with shaking at 200 rpm.

After the incubation period was finished the bacterial suspension was transferred into new falcon tubes (four tubes, 50 ml each) and centrifuged at 6000 x g for 40 minutes to collect the bacteria. Supernatant was then removed and pellet was washed by re-suspending in 10 of normal saline, centrifuged for another 40 minutes at 6000 x g. Supernatant was then removed and pellet re-suspended in 5 ml of ultrapure water. The

bacterial suspension was then disrupted by sonication (4 x 30 seconds with a 30 seconds interval on crushed ice in between) at 14 amplitude using an ultrasonic homogenizer (MSE-Soniprep-150, Labexchange, Germany).

Following the sonication, a final concentration of 2% (w/v) N-Lauroylsarcosine sodium salt was added to the sonicated cells and incubated for 30 min at room temperature, then the lysate was centrifuged at 6000 x g for 60 minutes to remove cell debris. After centrifugation, the supernatant was carefully removed to thick-walled Polycarbonate bottle, (355618 Konrad Beranek, Germany) and centrifuged at 32000 x g (45 min, 4 °C), (Beckman-Coulter Centrifuge; Optima L100XP). Supernatant removed and isolated OMP pellet was re-suspended in 300µl of ultra-pure water and stored at -20°C for further investigation.

2.8.4.13.3 Protein concentration determination using BCA assay.

BCA Protein Assay is a colorimetric detection method based on binding of bicinchoninic acid (BCA) with cuprous ion for the detection and quantitation of total protein in particular sample.

2.8.4.13.4 Standards and Working Reagent Preparation

The protein concentration in the OMPs was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK) following the manufacturer's protocol. In brief, BSA protein standards were prepared by suspending 0.002g/ml of bovine serum albumin in ultrapure water (2000µg/ mL) which was prepared according to dilution scheme for standard stated in the protocol and BCA Working Reagent was used as blank sample. Then serial dilutions were used to estimate the concentration of OMPs in the tested samples (working Range = 2,000µg- 20/Ml). The Pierce BCA working reagent is prepared by mixing 50 parts of BCA Reagent A (v/v) with 1 part of BCA Reagent B (50:1, Reagent A:B) and mixed to yield a clear, green working reagent.

2.8.4.13.5 BCA assay

In 96-Well Microplates, a volume of 25µL of each standard or sample were pipetted in triplicates followed by adding 200µL of the working reagent to each well. Plates were covered and mixed thoroughly on a plate shaker for 30 seconds and incubated at 37°C for about 30 minutes. After that, optical density, were measured at 562nm wavelength. ExcelTM was used to calculate the standard curve after subtracting the average of the measurements absorbance of the blank standard replicates from all of other standard and unknown sample replicate measurements. Then the protein concentration in the tested sample was calculated using the equation derived from the Pierce™ BCA standard curve.

2.8.4.14 SDS-PAGE

2.8.4.14.1 Resolving gel (12% Acrylamide)

Resolving gel stock solution (12%) was prepared following the manufacturer's instructions. For final volume of ten ml, 3.3 ml of deionized water, 2.5 ml of resolving buffer (EC-892, National Diagnostics) and 4 ml acrylamide solution (ProtoGel 30%; EC-890; National Diagnostics; Atlanta, GA, USA) was mixed together, and immediately before the experiment, 0.1 ml of 10% (w/v) ammonium persulphate (APS) (National Diagnostics) and 0.004 ml of N,N,N'N'-tetramethylethylenediamine (TEMED) (T3100, Melford) were added to the other components.

2.8.4.14.2 Stacking gel (3% Acrylamide)

The 3% stacking gel (2 ml) was obtained by mixing 0.25 ml of stacking buffer (EC-893, National Diagnostics), 0.33 ml of acrylamide solution (ProtoGel 30%; (EC-890; National Diagnostics) and 1.4 ml of deionized water, 0.002ml of N,N,N'N'-tetramethyl-ethylene amine (TEMED) and 0.02 ml of 10% ammonium persulphate (APS) (National Diagnostics)

2.8.4.15 Preparing the gel for SDS-PAGE analysis

SDS-PAGE was prepared as the following: the previously prepared 12% resolving gel (10 ml) was gently dispensed between two glass plates and About 2 cm space at the top of the glass plate was left for stacking gel. However, to avoid making bobbles, plate was covered with a layer of either dh_2o or isopropanol and allowed to completely solidify for about 30 minutes. If isopropanol was used on the top of resolving gel, after it has been removed when the gel polymerized, gel was washed with water and dried with clean paper towel. Afterwards, the 3% stacking gel mixture was poured on top of the solidified resolving gel and the comb was placed in the stacking gel, and allowed to polymerize at room temperature. Finally, gel was moved into an electrophoresis running apparatus after the comb was removed, and the chambers were loaded with 1X running buffer, Tris-glycine SDS-PAGE electrophoresis buffer TGS (EC-870; National Diagnostics).

2.8.4.16 Preparation of protein samples for SDS-PAGE

Twenty microliters of the purified OMPs were mixed with 10µl microliter of 2X Laemmli sample buffer (Sigma-Aldrich, UK). Then the mixture was incubated at 100°C for 10 minutes.

2.8.4.17 Gel electrophoresis of OMP samples

Five microliter of a molecular weight marker (precision plus proteinTM Dual color standards, BIO-RAD) pre-stained protein ladder was loaded in the two side wells, and 28µl of the heated proteins were loaded in the other wells. Samples were then electrophoresed for about 50 minutes at 200 V until the obvious dye reached to the bottom of the gel. The gels were then stained overnight using Coomassie Brilliant Blue stain (0.1% w/v) (R250; BioRad, UK) to visualise the OMP bands. Afterwards, gel was destained for1 hr in Coomassie destaining solution and repeated until the stains removed from the gel.

2.8.4.18 Analysis of the protein band patterns

The stained gels were visualized and images of the gel were saved as TIFF files format for further analysis. The molecular weight of the proteins in each band were predictably calculated using the protein standards marker (precision plus proteinTM Dual color standards, BIO-RAD). Cluster analysis of the different OMP profiles was performed using BioNumerics software, version 6. The Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) were used for cluster analysis

2.8.5 Tissue culture experiments

2.8.5.2 Human cell lines

2.8.5.2.1 Human non-malignant neonatal intestinal (H4) cell line

The H4 human fetal non-malignant primary small intestinal cell line, was derived from fetuses aged from 20 to 22 weeks of gestation, (Sanderson and Walker 1995). The cell line was used to represent immature Fetal enterocytes for evaluation as a new model of human epithelial cell lines by investigating virulence traits bacterial isolates thought to be responsible for neonatal infection. Cells were used between 6 and 20 passages.

2.8.5.2.2 Human colonic carcinoma epithelial (Caco-2) cell line.

Human colonic carcinoma epithelial cell line (Caco-2) was obtained from Nottingham Trent University cell cultures collection and stored in liquid nitrogen. This cell line was derived from a human colon adenocarcinoma. It is extensively utilised in studies of pathogen-host cell interaction because of its ability to develop a well-differentiated cell monolayer which is similar to small intestinal enterocytes, (Szymanski *et al,* 1995). Cells were used between 9 and 22 passages.

2.8.5.3 Tissue culture medium and buffers.

2.8.5.3.1 Growth medium for Human non-malignant neonatal intestinal (H4) cell line.

Dulbecco's modified Eagle's medium (high glucose pyruvate and L-Glutamine supplemented DMEM, 11995-073, Life Technologies, Gibco). Medium was supplemented with 10% Fetal Bovine Serum (Sigma, UK), 1% (v/v) non-essential amino acid solution 100x (15640-055, Life Technologies, Gibco, UK) and 1% (v/v) Penicillin-Streptomycin-Neomycin (PSN) Antibiotic Mixture (stock is 100 X, 15640-055, Life Technologies, Gibco, UK).

2.8.5.3.2 Infection medium for Human non-malignant neonatal intestinal (H4) cell line

500 ml Dulbecco's modified Eagle's was supplemented with 10% (v/v) Fetal Bovine Serum (F7524, Sigma), 1% (v/v) non-essential amino acid solution (15640-055, Life Technologies, Gibco, UK)

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

To prepare the growth medium for Caco-2 cells, 500 ml Minimum Essential Medium Eagle (MEM), (31095-052, Life Technologies, Gibco, UK) were supplemented with Fetal Bovine Serum (F7524, Sigma, UK) to a final concentration of 10% (v/v), and 1% (v/v) of non-essential amino acid solution (15640-055, Life Technologies, Gibco, UK). In addition, a final concentration of 1% (v/v) of Penicillin-Streptomycin-Neomycin (PSN) antibiotic mixture 100 X (15640-055, Life Technologies, Gibco, UK) was added to the medium.

2.8.5.3.4 Infection medium for Caco-2 cell line

To prepare the Infection medium, 500 ml of Minimum Essential Medium Eagle (31095-052, Life Technologies, Gibco, UK) was mixed with Fetal Bovine Serum (F7524, Sigma, UK) to a final concentration of 10% (v/v), and 1% (v/v) of non-essential amino acid solution 100x (15640-055, Life Technologies, Gibco, UK)

2.8.5.3.5 Dulbecco's Phosphate-Buffered Saline (DPBS)

Dulbecco's Phosphate-Buffered Saline 10X (14080-089 Life Technologies, Gibco, UK) with calcium and magnesium is a balanced salt solution used for a variety of tissue culture applications such as washing cells before dissociation after diluted to 1x with distilled water.

2.8.5.3.6 TrypLE™ Express Enzyme (1X), phenol red

TrypLE Express is a trypsin replacement recombinant enzyme (12605-028, Life Technologies, Gibco, UK) used for dissociating of a wide range of adherent mammalian cell lines. Appropriate volume was added to the confluent cells, and incubated for 5-10 min to detach cells from the growing surface.

2.8.5.4 Human cell growing conditions.

Cell lines used in this study (H4 and Caco-2) were recovered from liquid nitrogen. The frozen vials for both cell lines were defrosted rapidly and transferred to pre-warmed appropriate growth medium. Cells were then centrifuged at 1200 rpm for 5 min using CENTAUR2 centrifuge (MES, UK). The pellet was then re-suspended in 5 ml of appropriate growth medium and transferred to a new tissue culture flask (25 cm^3, Sarstedt/Germany) and total volume of approximately 10 ml, and incubated at 37ºC under 5% CO_2 until the about 90%-100% confluent monolayers were achieved. After they had reached confluence, cells were washed with PBS and then incubated at 37^oC for 5-10 min with 5 ml of TrypLE to release cells from the flask. Released cells were then centrifuged at 1200 rpm for 5 min and suspended in appropriate medium. Cells were either diluted in appropriate medium for maintenance twice weekly, and for adhesion, invasion, cytotoxicity or IL-8 assay, using haemocytometer, 1 ml was seeded in 24 well plates to a concentration of 2 x 10^4 cells/ml of Caco-2 cell line and 1 x 10^5 cells/ml for H4 cell line

2.8.5.5 Optimization of experimental conditions.

2.8.5.5.1 Cell line growth time required.

Both cell lines were optimized in regard to the time required to reach up to >90% confluent in 24-well plates. Cells were seeded according to the recommended protocols and the increase of the number of cell per ml determined over a period of 48h in time points 5, 8, 12, 24, 48 after seeding. The number of dead cells was also determined by using Trypan blue, as healthy cells appear bright while dead cells will absorb the dye.

2.8.5.5.2 Effect of passage number on invasive ability of the bacteria

This experiment was designed to investigate the role of passage number in the invasion of selected bacterial strains. High passage number (48-51) and low passage number (17-19) cells of H neonatal cell line was used in this assay. Cells were prepared as described in section 2.5.8.4. After cells reached confluence in 24-well plates, bacterial strains were adjusted to $OD6_{00}$ and co-incubated with the cells for three hours. Medium was then removed using Vacusafe Aspiration System (158 310, Integra Biosciences) and gentamycin protection assay was then performed. The number of internalised bacteria to each passage determined by Miles and Misra method and compared.

2.8.5.6 Bacterial Adhesion to H4 and Caco-2 cell lines.

Human H4 and Caco-2 cell lines were grown in DMEM medium. After confluence was observed, wells were washed three times with DPBS. Bacterial strains from 16-20hrs TSB culture were adjusted to OD_{600} 0.05 in appropriate pre-warmed infection medium (overnight incubated at 37^{o}C for purity), and 1 ml added to each well in 24-well plate. Cells were then incubated for 3 h at 37^{o}C under 5% CO_2 in the tissue culture incubator. Next, medium was removed using Vacusafe Aspiration System and wells washed 3 times with DPBS. Cells were then soaked with 200 µl of 1% (v/v) Triton X-100 and pipetted many times to lyse the human cells. Lysates were serially diluted in PBS and Miles and Misra techniques used on TSA plates to determine the viable count as a Colony Forming Unit (CFU). After invasion assay was carried out, the total number of internalized bacteria was

subtracted from the number of bacteria obtained from this assay to determine the number of adhesive bacteria.

2.8.5.7 Bacterial invasion of H4 and Caco-2 cell lines (Gentamicin protection assay)

For internalized bacteria, a gentamicin protection assay was performed. H4 and Caco-2 cell lines were prepared as described in section 2.8.5.4 and after confluence reached, wells were washed three times with DPBS and infected with selected bacterial strains at OD_{600} of 0.05. Plates were incubated for 3 h at 37ºC under 5% CO_2, then medium was removed as described in adhesion assay followed by three times washing with DPBS. In order to kill all of the non-internalised bacteria, one ml of tissue culture medium containing 125 µg/ml gentamicin was added to each well and re-incubated in same condition for another hour.

Subsequently medium were removed and cells washed 3 times and lysed with 1% (v/v) Triton X-100. Lysates containing internalized bacteria were serially diluted in PBS and Miles and Misra technique used on TSA plates to determine the viable count as a Colony Forming Unit (CFU)/ml.

2.8.5.8 Patterns of bacterial adhesion with H4 cell line human epithelial cells.

In order to visualize bacterial attachment to the human cell line, cells were seeded in six well plates containing sterile coverslips. Plates were incubated for 24h at 37^{o}C under 5% CO_2, and then washed 3 times with DPBS and infected with selected bacterial isolates at OD_{600} 0.05 for 3h incubation period, then the medium was aspirated and cells washed again 3 times in DPBS. To fix bacteria, 4 ml of 100% methanol was added and left for about 5 minutes and then removed with vacuum pump. Coverslips were then submerged in Giemsa stain (5% v/v) for 30 min, then washed with distilled water and left to dry at 21^{o}C. Light microscopy was used to visualize the pattern of attachment under oil immersion lens 100x. Non- infected standard wells were used as a control in this experiment.

2.8.5.9 Translocation assay.

2.8.5.9.1 Preparing polarized monolayer H4 and Caco-2 cells.

Bacterial translocation through polarized monolayer H4 cells was investigated. Human cells were seeded at concentration of about 1 x 10^6 on the upper chamber of 6.5 mm Transwell with 3.0 µm pore polycarbonate membrane insert (CLS3415 Sigma, UK). Medium was changed three times a week and Trans Endothelial Electrical Resistance (TEER) was measured weekly starting from the third week, as there was no notable increase in the TEER of H4 cells inserts in the first three weeks. Assay was conducted when TEER reached to average of 220Ω or above and inserts which did not reach the desired TEER were excluded. Polarization was also confirmed using blue dextran assay. In brief; 200µL of 10mg/mL dextran blue (FD150-Sigma, UK) diluted in DMEM medium were added to the apical side of the inserts, and 1mL of the appropriate fresh medium was added to the basolateral side. After 2hrs incubation at 37◦C, in 5% CO_2 incubator, 100uL of medium was transferred from the basolateral chamber to 96-well plate in triplicates and analysed for the presence of dextran blue using a microplate reader (BioTek, UK) at 600nm wavelength.

2.8.5.9.2 Bacterial translocation.

Bacterial isolates (n=12) including negative and positive controls were grown on TSA for about 24h and the one colony transferred to 5 ml of TSB and incubated for about 16-24h at 37ºC in shaking incubator. Afterwards, cultures were adjusted to OD_{600} of 0.05 in appropriate tissue culture medium. 300µl were added into the upper chamber and 1ml free antibiotic medium into the basolateral side. Bacterial translocation was examined after 1 and three hrs, 100 µl of the medium were collected from the lower side each time and were for viable counting on TSA plates.

2.8.5.10 Cytotoxicity of bacteria to human cell lines.

2.8.5.11 Trypan blue cytotoxicity assay.

The cytotoxic activities of selected bacterial strains were investigated. Bacterial isolates were co-cultured with H4 and Caco-2 cell lines as described previously in adhesion assay with some modification. Briefly, after 3 hours incubation period at 37^{o}C under 5% CO_2, medium from each well was collected since there was an expectation of the presence of unattached dead cells in the medium. The remaining cells were detached by addition of TryplE to the wells for 10 min to detach the cells which were then combined with those collected by centrifugation previously. These combined cells were centrifuged for 5 min at 1200 x g, and the pellet resuspended in 100 µl of growth medium. Cell viability was determined using Trypan blue dye (8154-20ML SIGMA, UK), 30 µl of cell suspension were mixed with equal volumes of Trypan blue in 96-well plates and then 20µl transferred to a haemocytometer counting chamber. Live cells appeared bright and dead cells appeared as blue coloured because of the damage in the cell membrane permeability and binding of the dye to the cell components. Blank wells cultured with normal growth medium, and the results were calculated as fold of the blank.

2.8.5.11.1 Lactate dehydrogenase assay.

Twenty-nine strains were used in this assay. This experiment was performed as described by Castiaux *et al,* (2016) with minor modification and aimed to confirm the results of Trypan blue cytotoxicity assay. Lactate dehydrogenase assay kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay-G1780 Promega, UK) was used. Assay was performed according to the supplier's protocol. Briefly; H4 and Caco-2 cell lines were prepared for the recommended concentration and in suitable medium, and then 100µl/well were cultured in 96-well plates. Cells were incubated in tissue culture conditions until confluent obtained and then used for further steps. The selected bacterial isolates from -80^{o}C stock were streaked on TSA plates and incubated 16-24h, and then one colony inoculate into approximately 5 ml of fresh TSB, and incubated in shaking incubator (200rpm) for 16-24

hours. Bacteria were adjusted to OD_{600} 0.05 in suitable tissue culture infection medium (section: 2.8.5.3.4) and one hundred microliters were added to the cells in triplicates.

Human cell lines were then incubated with the bacteria for three hours in tissue culture conditions. After the incubation period plates were centrifuged at 2500 x g for 5 min in order to obtain cells from supernatant cell (Castiaux *et al,* 2016). Then 50 µl of the supernatant was removed to new 96-well plate and 50 µl of the CytoTox 96 reagent was added to each sample aliquot and incubated for 30 min. Ater that, 50 µl of stop solution were added to each well of the 96-well plate. Cytotoxicity was determined using plate reader to record the absorbance at 490nm.

Three controls were used in this assay, for the maximum LDH (positive control), 100 µl/well of fresh medium (without bacteria) were added to three well of the chosen cell line, and then 10µl of 10X lysis solution were added to these wells 45 minutes before the end of the assay. For the negative control, only 100µl of medium was added to three well of the chosen cell line. For the medium background, 3 wells containing only medium without any cells were used.

2.8.5.11.2 Cytotoxicity of bacterial outer membrane proteins to human cells

Bacterial OMPs were investigated in regard to their role in host cells proliferation and increasing the cells permeability throughout damaging host cells membrane or directly killing of host cells. Extracted OMPs at concentration of 100µg/ml in appropriate tissue culture medium were incubated with human cells for 3h at 37^{o}C in 5% CO_2. Cytotoxicity of the selected OMPs was then determined using LDH assay as described in section 2.8.2.11.1.

2.8.5.12 Determining the virulence factors behind high cytotoxicity of *C. sakazakii* ST3 strain 978 and *E. coli* K1 strain 939.

2.8.5.12.1 Effect of chloramphenicol on bacterial cytotoxicity.

This assay was designed to investigate if the production of new proteins is required for the high cytotoxicity observed by *C. sakazakii* ST3 strain 978**.** H4 and Caco-2 cells were seeded in 24-well plates, and after confluence obtained cells were then incubated with selected bacterial isolates in presence of 20µg/ml Chloramphenicol antibiotic for 3h at 37^{o}C in tissue culture incubator. Following the incubation period, supernatant was collected, and human cells incubated with 0.2 ml of TryplE for 10 minutes in tissue culture incubator. Cells were collected from the wells after repeated pipetting to insure completely removal of attached cells, and then added to the supernatant collected from each well. This suspension was centrifuged for 5 minutes at 1200x xg to collect the cells. To determine bacterial cytotoxicity, Trypan blue assay was performed to count living and dead cells.

Non-treated bacteria were used as a control in this experiment to compare the growth performance of investigated strains before and after adding chloramphenicol.

2.8.5.12.1.1 Effect of chloramphenicol on bacterial growth.

Two hundred microliter from the bacterial suspension used in chloramphenicol assay (OD_{600} of 0.05), (with and without chloramphenicol) were transferred into 96-well plate and serially diluted in TSB medium up to 10^{-11}, and then incubated for 16-24 hours. To compare results, after 3h incubation, 200µl from bacteria with chloramphenicol and 200µl from bacteria without chloramphenicol were also serially diluted in 96-well plate up to 10^{-11} and incubated for 16-24h. After that, the log of bacterial growth was recorded from the maximum dilution of each strain and compared with the initial values.

2.8.5.12.2 Investigation of the role of direct contact of high cytotoxic *C. sakazakii* 978 on cytotoxicity

This experiment was designed to investigate whether bacteria need to be physically in contact with human cells, or if the cytotoxicity is due to secreted proteins. Two different assays were made to investigate this.

2.8.5.12.2.1 Role of secreted proteins on bacterial cytotoxicity.

Bacterial isolates from -80°C freezer were streaked on agar plates and after 16-24h 1 colony was inoculate into 5ml of TSB medium, and incubated for 18 hours at 37°C in shaker. Bacterial strains were then adjusted to OD_{600} of 0.05 in DMEM medium and re-incubated for 5h. Next, the bacterial suspension was centrifuged for 5min at 2000 x g and filtered through 0.45µm syringe filter followed by another filtration through 0.2µm syringe filter to insure no contamination with bacterial cells. One ml of this filtrate was added to human cells per well and incubated in tissue culture incubator for 3h. Filtrate cytotoxicity was determined using Trypan blue assay.

2.8.5.12.2.2 Role of physical contact in the high cytotoxicity of *C. sakazakii* 978.

The role of direct contact in *C. sakazakii* strain 978 cytotoxic was also investigated using no direct contact assay. This assay is based on the idea of adding the bacteria in the upper chamber of 0.3 µm polycarbonate trans-well inserts. Briefly, human cells after reaching confluence in 24-well plates were washed twice with DPBS. After that, 0.3µm trans-well inserts were placed on the wells and bacteria after it has been adjusted to OD_{600} of 0.05, carefully 250 µl were added into the upper chamber. Wells were then incubated for three hours in tissue culture incubator. Next, bacterial cytotoxicity was obtained using Trypan blue visualisation method.

2.8.6 Effect of eukaryotic cytoskeleton inhibitors on bacterial invasion.

The capability of pathogenic bacteria to attach to host tissues is a key factor, as this represents an early phase of host/pathogen contact *in vivo*. This binding is also vital in

some instances for host cell invasion. To investigate the role of the eukaryotic cytoskeleton in bacterial invasion to H4 and Caco-2 cells lines, an invasion experiment was conducted by pre-incubating the human cell lines with different microfilament (MF) and microtubule (MT) inhibitors. The inhibitors were Cytochalasin D, Colchicine and Nocodazole which were obtained from Sigma, UK. Pre-incubation of human cells occurred as follows: Cytochalasin D (2 µM) for 30 min at 37°C, Nocodazole (20 µM) and Colchicine(10 µM) were pre-incubated with epithelial cells for 60 min at 4°C followed by half an hour at 37°C before addition of the bacterial strains (Oelschlaeger *et al,* 1993 & Mohan Nair and Venkitanarayanan, 2007). After the pre-incubation period with each inhibitor finished, selected bacterial isolates were added to the cells. Plates were then incubated for 3 h at 37°C and 5% CO_2, and then washed three times with DPBS followed by one hour incubation in presence of gentamicin antibiotic (125 µg/ml) under the same conditions. After gentamycin protection period finished wells were washed 3 times with DPBS and Miles and Misra method was applied for the invaded bacteria. For the control, untreated cells were incubated without addition of any inhibitors and incubated in the same conditions with selected bacterial strains. The effect of these inhibitors was then determined by a difference between invasion with and without inhibitors. Results calculated using the following formula to obtain the relative invasion:

$$\text{Relative invasion} = \frac{\text{number of internalised bacteria with inhibitors}}{\text{number of internalised bacteria without inhibitors}} \times 100$$

Then results were expressed as a percentage of the invasion without adding any inhibitor.

2.8.7 Inflammatory response of human cells to bacteria challenge.

2.8.7.1 Interleukin 8 production by non-polarized monolayer human cells

2.8.7.1.1 Preparing IL-8 samples

Previous findings suggested that the inflammatory nature of intestinal enterocytes plays a critical role in occurrence and development of disease, therefore, we investigated the

immunological response of our H4 cell line when incubated with bacteria compared with the old model Caco-2 cells. Human cells were seeded in 24-well (section 2.8.2.3) and incubated for 24h until confluence was observed, then wells were washed three times with DPBS, and new medium without Foetal Bovine Serum added to each well and incubated for another 24 h. Afterward, human cells were co-cultured for 3h with selected bacterial strains in medium without FBS at a concentration of OD_{600} 0.05. Supernatants were then collected and centrifuged at 1200 x g to remove bacterial cells and stored at -20°C until analysis. H4 cells were also co-incubated with Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 at a concentration of 50 and 100µg/ml (L6529-1MG, Sigma), and 1 ng/ml human interleukin 1 beta (IL-1β/Sigma. UK) at same condition as positive controls for inducing IL-8 release whereas wells incubated with FBS free medium alone were used as a blank for this assay.

2.8.7.1.2 Enzyme-Linked Immunosorbent Assay (ELISA) for IL-8 detection.

Production of the pro-inflammatory Interleukin-8 (IL-8) in response to bacterial challenge was investigated using Human IL-8 ELISA Ready-SET-Go 2nd Generation (eBioscience, UK). According to the supplier instructions, the capture antibody was diluted to the recommended dilution and 100 µl incubated overnight in Nunc MaxiSorp® flat-bottom 96 well plate (eBioscience, UK), sealed with 96-well microplate sealing tape to avoid reagent evaporation. Next day, wells were washed three times with prepared washing buffer and blocked with 200µl 1X ELISA/ELISPOT Diluent for 1hr, washing repeated and plate blotted on clean tissue paper to remove any residual buffer. IL-8 standard was serially diluted for 8 different concentration points starting from 250 pg/ml to 4 pg/ml, then 100 µl/well of either standard or sample was added to the 96 well plate and incubated for 2 hours at 21°C after which it was sealed again with 96-well microplate sealing tape.

Subsequently the wash step was repeated and 100µl of detection antibody was added and incubated for another 1h, and wells then washed again. Thereafter 100 µL of the working dilution of Avidin-HRP were added to each well and left at 21°C for 30 minutes. during this step, the plate was covered with aluminium foil to avoid the effect of direct light. Subsequently a final wash step was completed and plate was incubated for another

15 min with 100 µL of 1X TMB solution in each well, avoiding direct light. After 15 min, 50 µL of Stop Solution (sulphuric acid 1M) was added to each well without washing, the plate then was gently agitated to ensure thorough mixing and the optical density was taken using a microplate reader at 450 nm then readings at 570 nm subtracted from the readings at 450 nm as recommended by manufacturer's instructions.

2.8.7.2 Role of apical activation of polarized human cells on the IL-8 production

Polarized monolayer of human cells (section 2.8.2.8) were apically activated by adding 300 µL of bacterial suspension (density of OD_{600} 0.05) from selected bacterial isolates (n=12), and incubated for 3 h at 37°C and 5% CO_2. Samples were collected at 1 and three hours from both upper (50 µl) and basolateral chambers (100 µl) for IL-8 analysis (section 2.8.7.3).

2.8.7.3 IL-8 production response to purified bacterial outer membrane proteins (OMPs).

Bacterial OMPs after determining the protein concentration, were added to H4 and Caco-2 human cells to a concentration of 100µg/ml. Briefly, H4 and Caco-2 human cell lines were seeded according to each protocol and incubated in 37°C at 5% CO_2 until about 100% confluent obtained. Then wells were washed twice with DPBS and re-incubated with appropriate medium in absence of FBS for another 24h. Afterwards, bacterial OMPs were adjusted to concentration of 100µg/ml in DMEM without FBS and one millilitre per well was added in triplicates. Wells were then incubated for 3 hours in tissue culture incubator. Supernatant were then collected and stored at -20°C. IL-8 analysis was conducted using human IL-8 ELISA (section 2.8.7.2) according to the supplier instructions.

2.8.7.4 Multiple Cytokine and Growth Factors Magnetic Luminex® Assays

2.8.7.4.1 Reagents preparation.

2.8.7.4.1.1 Preparing 1X Antibody Beads.

The 10x concentrated antibody beads were vortexed for 30 seconds, and 1X stock was prepared by diluting 2.5 µL of 10X beads in 25 µL of Working Wash Solution for each well. For 96-well plate, a Volume of 0.3 mL of 10X antibody bead concentrate mixed with 3mL of the working wash solution.

2.8.7.4.1.2 Preparing 1X Biotinylated Antibody.

Working solution of 1X Biotinylated Antibody stock was prepared by diluting 10 µL of 10X Biotinylated Antibody in 100 µL of Biotin Diluent for each well according to the supplier recommendations. This step was done in about 15 minutes prior to use.

2.8.7.4.1.3 Preparing Streptavidin-RPE

Working solution of 1X Streptavidin-RPE stock was prepared by diluting 10 µL of 10X Biotinylated Antibody in 100 µL of Biotin Diluent for each well according to the supplier recommendations. This was done in at least 15 minutes prior to use. Streptavidin-RPE was protected from exposing to the direct light during handling and experimental steps.

2.8.7.4.1.4 Preparing wash solution.

20X Wash Solution Concentrate was warmed to 37°C and mixed until the precipitate was dissolved. 1X Working Wash Solution for use with a 96-well plate was prepared by mixng 1 part of 20X Working Wash Solution concentrate with 19 parts of deionized water and mixed properly in a 1000 mL container. The prepared 1X working wash solution was stable for up to 2 weeks when stored at 2–8°C.

2.8.7.4.2 Sample preparation.

Human cells were seeded in 24-well plate according to each protocol and incubated in 5% CO_2 incubator at 37^{o}C until confluence was obtained. Bacterial strains were prepared as described before and adjusted to OD_{600}nm of 0.05, and incubated with human cells for 3h in tissue culture incubator. After that supernatant was collected and centrifuged 1200 x *g* for 10 min and stored at -20^{o}C immediately after centrifugation.

2.8.7.4.3 Reconstituting lyophilized standards done

As recommended by supplier, standards were reconstituted in 50% Assay Diluent and 50% inflammation infection medium which do not contain FBS or antibiotic within 1hour prior to performing the assay. The standard vials were allowed to stand undisturbed for 10 minutes, and gently swirled and inverted 2–3 times to ensure completely reconstitution of the contenes and incubated at room temperature for an extra 5 minutes.

2.8.7.4.4 Standard curve

To prepare a standard curve, the reconstituted standard was serially diluted in a mixture of 50% assay diluent and 50% tissue culture medium and mixed by gently pipetting 5–10 times. Standard curves were generated automatically by the Luminex xMAB technology system.

2.8.7.4.5 Preparing plate with magnetic antibody beads.

Due to rapid settling of magnetic beads, it was well mixed immediumtely prior to use for about 30 seconds, then 25 µL of the 1X antibody bead solution were pipetted into each well and plate kept protected from the light because the fluorescent beads are light sensitive. Subsequently, 0.2ml of the working wash solution was added to the wells and beads allowed to soak for about 30 seconds. Wells were then washed twice with 200µL of working wash solution.

2.8.7.4.6 Magnetic Separator washing Method:

After beads and working wash solution been added into the provided 96-well Flat Bottom, plate were then placed onto the magnetic separator and incubated for about 2 minutes to immobilize the beads on the bottom of the plate. After that, plate were hold securely together with the magnet and fluids were inverted out. Subsequently, plate was then removed from the magnet and 0.2 ml of the working wash solution was added to the wells and beads allowed to soak for about 60 seconds. Plate was further placed onto the magnetic separator and incubated for about 1 minute to immobilize the beads and again plate was hold securely together with the magnet and wash solution was removed followed by blotting the plate on a clean paper towels several times. The plate then was removed from the magnet, for additional washing, and the above steps were repeated.

2.8.7.4.7 Assay procedure

After first wash, a volume of 50 µL incubation buffer was pipetted into each well, and 100 µL of an appropriate standard dilution were added to wells in the first two columns for the standard curve. Then, 50 µL of a mixture of 50% assay diluent and 50% tissue culture medium v/v were added to the other wells to be used for the samples followed by adding 50 µL of the samples to the appropriate wells. The microplate was covered with aluminium foil- and incubated for 2 hours at room temperature on an orbital shaker at 550 rpm. Afterwards, the plate placed was into the magnet separator and liquid removed from wells. Plate carefully removed from the magnetic separator and washed by adding 200 µL of washing solution to the wells, and beads allowed to soak for 90 seconds. Then the plate was placed again into the magnet and washing buffer removed from the wells. This was repeated twice, after the second wash, plate was blotted on a clean paper towel to remove the remaining liquid.

Working solution of 1X Biotinylated detector antibody was prepared about 15 minutes prior to use. A hundred microliters of prepared biotinylated detector antibody were added per well and the plate incubated on an orbital shaker for 1 hour at room temperature.

After one hour, liquid was removed from the wells using magnetic washing equipment and wells washed twice. Subsequently, 100 µL of prepared 1X Streptavidin-RPE were added to the wells, and incubated at room temperature for half an hour on an orbital shaker at 550 rpm. After the incubation period Streptavidin-RPE was removed from wells and wells washed as mentioned before three times.

Finally, beads were re-suspended in 125 µL of working wash solution and placed on an orbital shaker for 2–3 minutes. Plate was then read using the Luminex xMAB technology system and the concentration of each human cytokine and growth factors in the samples was determined from the standard curve using curve fitting software.

2.8.8 Gene expression study.

2.8.8.2 RNA Isolation and Purification from Adherent Culture Cells Protocol

2.8.8.2.1 Seeding of human cells for RNA infection and RNA isolation

Caco-2 and H4 human cells were seeded in 25 cm² tissue culture flasks by adding 8 ml at concentrations of $2X10^4$ and $1X10^5$ respectively. H4 cell line was used after 24 hours and the number of cells used in this assay was 1.98 X 10^6, while Caco-2 cells were used after 48 hrs from the seeding and the number of cells 2.4 X 10^6, which was calculated from spare flasks using Countess II FL Automated Cell Counter (Thermo Fisher, UK).

2.8.8.2.2 Infection with bacteria.

Prior to this experiment, tissue culture flasks were washed twice with DPBS and 8 ml of bacterial suspension were added at density of OD_{600} 0.05, followed by three hours incubation (Furrie *et al,* 2005). After that bacterial suspension was removed and cells washed twice with ice cold DPBS. Standard control wells were incubated with fresh medium.

2.8.8.2.3 Human cells harvesting

Human RNA was extracted using SV Total RNA Isolation System (Promega/ Z3100). Briefly: Human Cells were detached from the flasks using cell scraper in 1.5 ml ice cold DPBS, then transferred into 1.5 Eppendorf tube and centrifuged at 1500rpm for 5 min. Supernatant was removed and Eppendorf tubes were inverted on a sterilised towel paper to remove any residue liquids.

2.8.8.2.4 Lysing of the human cells for RNA isolation

After centrifugation, 175 µl of RNA Lysis Buffer were added to the cell pellet and mixed by pipetting several times until cells werecompletely suspended. Cell lysate was then homogenized using 20-gauge (0.9 mm) needle (BD Precisionglide® syringe needles 20-gauge, Scientific Laboratory Supplies */Z118052)* attached to a sterile plastic syringe and passed through for 5–10 times. Subsequently, 350µl of RNA Dilution Buffer (blue) were added to 175µl of lysate and mixed by inverting the tube 3–4 times and placed in heating block at 70°C for 3 minutes. Tubes were then centrifuged at 13000 x g for 10 minutes at 20–25°C and the cleared lysate (supernatant) carefully transferred to a new fresh micro-centrifuge tube using pipette to avoid disturbing the pelleted debris. 200 µl 95% ethanol were added to the cleared lysate, mixed by pipetting 3–4 times and transferred to the spin column and centrifuged at 13000 x g for one minute. Flow-through was removed and spin columns were washed with 600 µl of RNA wash solution and centrifuged at 13000 x g for 1 minute. The collection tubes were emptied from the washing solution and placed in a rack, and 50 µl of DNase incubation mix were then added to the middle of the columns and Incubated for 15 minutes at room temperature. After that, 200 µl of DNase stop solution were added and centrifuged at 13000 x g for 1 minute followed by washing with 600µl RNA wash solution (with ethanol added) and centrifuged for 1 minute at 13000 x g . washing step was repeated with 250 µl RNA wash solution (with ethanol added) and centrifuged at high speed for another 2 minutes. Spin columns were then transferred into the collection tubes, and 100 µl nuclease free water were carefully added to the middle of the membrane and centrifuged at 13000 x g for 1 minute. Spin columns discarded and purified RNA was then proceeded for further purification.

2.8.8.2.5 RNA Purification using DNA-*free*™ Kit

The RNA was further purified using DNA-*free*™ Kit for RNA Purification (Thermo Fisher Scientific, AM1906) according to manufacturer's instructions. In brief, 10% of the total volume of 10X DNase I and 1 µL rDNase Buffer I was added to the RNA samples and incubated at 37°C for 30 min, then DNase inactivation reagent approximately 10% (v/v) of the whole volume were added and mixed well. This mixture was incubated for about 2 min at room temperature and occasionally mixed to ensure re-dispersal of the DNase inactivation reagent. After that samples centrifuged at 10000 x g for 1.5 min and supernatant, which contains the RNA, were carefully transfer into a new tube. Purified RNA was then quantified by a NanoDrop-2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at –70°C for downstream analysis.

2.8.8.2.6 Reverse Transcription.

Purified RNA was converted to cDNA by using iScript™ cDNA Synthesis Kit (Bio-Rad–1708891) according to the supplier recommendation. In brief; on ice, 1 µg of the experimental RNA was combined with 4µl of 5x iScript Reaction Mix containing a blend of random hexamer primers and oligo-dT. This was mixed with 1µl of iScript Reverse Transcriptase and nuclease-free water was added to final volume of 20 µl. For this experiment the final volume for each component was increased twice to get enough product for q-PCR (2 µg RNA, 8 µl of 5x iScript Reaction Mix, 2 µl of iScript Reverse Transcriptase and then volume made up to 40 µl). Following, the reaction mix was incubated in thermal cycler (C1000 Touch Thermal Cyclers from Bio-Rad) for 5 min at 25^{o}C for priming step followed by 20 min incubation at 46^{o}C for reverse transcription followed by heat inactivation for 1 min at 95°C.

2.8.8.3 Gene expression and Quantitative Real Time Polymerase Chain Reaction (q-PCR).

2.8.8.3.1 Selected genes and primer design

Human Toll-like Receptors (Human-TLRs)1-10 and Nuclear Factor NF-κB genes expression in response to exposure to bacterial infection was investigated. Human ten TLRs set was purchased from Invivogen Company, but unfortunately, they did not provide any information about the sequence. Information about the primer set can be found in the following link: http://www.invivogen.com/htlr-primer-. NF-κB primers were carefully designed using PUBMED and primer3 for primer design. About 200 nucleotides were copied from the joint point of the last two exons and then copied to primer3. From the library, human was chosen with product size of 80-120, and parameters of q-PCR was adapted. The primer sequences are showing in table 2.4.

Table 2-2 primers for human housekeeping genes and a NF-κB.

Gene	Primer Sequence	Reference
GAPDH	ATGTTCGTCATGGGTGTGAA	The recent PhD study
	GGTGCTAAGCAGTTGGTGGT	
β-actin	CCCAGCACAATGAAGATCAA	The recent PhD study
	ACATCTGCTGGAAGGTGGAC	
NFKB subunit 1	CCAGTGAAGACCACCTCTC	The recent PhD study
	TCGGAGCTCGTCTATTTGCT	
NFKB subunit 2	GACACACCACTGCACCTAGC	The recent PhD study
	GCGTGGTGGATGACATAGACT	

Human housekeeping genes Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta-Actin (β-actin) were used to normalize the investigated genes.

2.8.8.3.2 Quantitative reverse transcription PCR (RT-qPCR)

The ifferences in gene expression in response to bacterial infection were validated using qRT-PCR. The reaction was performed in thin wall 384 well plate (Bio-Red, HSP3805) and total volume of 5 µl per well. Each reaction mixture contains 0.2 µl of primer set mix, 2.5 µl SYBR Green GoTaq® qPCR Master Mix (Promega, A6001) and 2.3 µl from cDNA product (diluted in nuclease free water 1:10 before use to avoid interference from the RT reagents in qPCR, and to have enough sample for qPCR) and each primer was performed in quadruplicate and experiment repeated twice. A mixture of no cDNA templet added was used as a negative control in this assay and 2.3 µl of nuclease free water were added to control wells. Plate then sealed with PCR Plate Sealer (Bio-Red, 1814000) and centrifuged at 1000 x g for 2 min to ensure collection of all components and placed in a CFX384 Touch™ Real-Time PCR Detection System (Bio-Red). The following parameters were used in this assay as shown in table 2-5.

Table 2-3 parameters of q-PCR used in this project.

	Actin / step	Cycles	Cycling Program
	Hot-Start Activation	1	95°C for 2 minutes
	Denaturation	40	95°C for 15 seconds
	Annealing/Extension*		60°C for 60 seconds
	Dissociation	1	60–95°C

* Data collected during the annealing step of each cycle.

After the run was completed, data was analysed using the CFX Manager Software.

Chapter 3. Phenotypic and physiological characteristics of bacterial isolates.

3.1 Introduction

Neonates are infants aged between 0 and 28 days (WHO 2015). This group of young and often low birth weight birth children, are at increased risk of infection by a variety of pathogenic microorganisms including bacteria (Levy *et al,* 2004; Alkeskas *et al,* 2015) often from meals such as breast milk and reconstituted infant formula. Despite being in sealed containers, powdered infant formula is never sterile and there have been reports that indicated that infant powder formula is contaminated with various pathogenic bacteria such *as Cronobacter sakazakii, Klebsiella oxytoca, Staphylococcus aureus, Escherichia coli* O157, *Salmonella enterica, Klebsiella pneumoniae, Serratia marcescens,* and others (Wang *et al,* 2009; Kalyantanda *et al,* 2015; Parra-Flores *et al,* 2015 and Boo. 2016)..

The neonatal skin and intestine are considered to be sterile prior to birth, and the colonisation of neonatal skin and mucosal surfaces by the majority of microbial flora is attained from the new-borns' mother at delivery and proceeds for several years to establish the gut microflora (Dickson, 2009; Arumugam *et al,* 2011).

Bacteria are the main causative of neonatal infection. Enterobacteriaceae family is considered one of the most common causes of neonatal infections including *E. coli* and *Klebseilla* species that are among the most important pathogens responsible for serious disorders in neonates (Najeeb *et al,* 2012).

3.1.1 Bacterial identification.

A rapid and accurate identification of bacteria is essential when preventing, treating or investigating an infection. For neonates at risk, this is even more important due to their highly vulnerable state, and there is evidence that food contaminated by pathogenic bacteria is responsible for some neonatal pathology (Angulo *et al,* 2008). There are different phenotypic and genotypic procedures that can be applyied in order to identify

and classify bacterial isolates (Patel *et al,* 2012). The initial identification of bacterial isolates is mostly based on physiological and chemotaxonomic characters of the bacterial strain as well as the phenotypic properties (Patel *et al,* 2012).

Colony morphology is a very important feature in bacterial identification. For example, *Cronobacter* was firstly known as "yellow pigmented *Enterobacter cloacae*" bacteria before becomes classified as *Enterobacter sakazakii* and finally *Cronobacter* species (Farmer, 2015). Description of bacterial colony based on the binding of bacterial cells to specific dyes was used in phenotypical characterization of *Salmonella* (Finn *et al,* 2013) and *Cronobacter* spp. such as Congo red which is one of the common use in morphological featuring (Yan *et al,* 2015).

Daskaleros and Payne (1987) proposed that binding of Congo red to cell surface of *Shigella flexneri* and *E. coli* is an indicator of the ability of bacteria to bind hemin and protoporphyrin IX which are required for the oxygen-carrying capacity of haemoglobin (Palma *et al,* 1994). Binding to Congo red is also used as evidence of curli fimbriae expression by *Salmonella* Isolates (Finn *et al,* 2013) which might indicate to bacterial motility and biofilm production as well as the ability to attach to the host cells. Yan *et al,* (2015) found that different isolates of *C. sakazakii* expressed different phenotypes based on their binding to Congo red and strains that exposed more binding to Congo red also showed greater motility and biofilm production. Similarly, this manner of phenotypic characterisation was also described by Finn *et al,* (2013) for different *Salmonella* isolates. Therefore, the characteristics typically used for identification should be examined to determine if they are differentially expressed by *Cronobacter* isolates linked to specific pathologies in this study.

Many biochemical kits such as API20E and ID32E are also applicable techniques that are widely used to identify both pathogenic and non-pathogenic bacteria. However, Jackson and Forsythe, (2016) demonstrated that some available biochemical kits are not sufficient to identify bacterial isolates to the genus level such as *Cronobacter*, and only 80% of isolates were identified to the genus level using databases for either the ID32E or API20E test kits.

One of the most accurate identification method is Multilocus Sequence Typing (MLST) molecular technique that based on the sequence analysis of multiple housekeeping genes that distributed across the bacterial genome.

This molecular technique can be successfully used for an accurate identification of some pathogenic bacteria such as *Cronobacter* to species level based on seven housekeeping genes, which are *atpD, fusA, glnS, gltB, gyrB, infB*, and *ppsA*. *Cronobacter* MLST database has been established and has open access at http://www.pubMLST.org/cronobacter (Baldwin, *et al,* 2009; Joseph & Forsythe, 2012). This scheme has been applied for different bacterial isolates including *Klebsiella pneumoniae* and *Cronobacter* spp. and provided unambiguous results which might be useful for the epidemiology of different pathogenic bacteria (Diancourt *et al,* 2005; Baldwin, *et al,* 2009).

3.1.2 Growth rate

The growth rate of pathogenic bacteria might be one of the key factors of its pathogenicity. The rapid growth rate of specific bacterial isolate may explain why it is the dominant or commonest causes of particular infection, and better understanding of its ecology is very important to control the growth or to eliminate pathogens (Anderson *et al,* 1979; Beuchat, 2002).

The population growth of specific bacterial isolate can be investigated by studying the growth curve of a bacterial culture. This can be done by cultivation of microorganism under condition of fixed amount of substrate, which is known as a batch culture, or continuous culture when the amount of available substrate remains the same during the experiment. However, when growth is achieved in batch culture and no fresh medium is provided, nutrient concentrations decline and concentrations of waste increase (Ingraham *et al,* 1983; Maier *et al,* 2000). This usually lead to the competition between bacteria and produce toxic compounds to dominate the environment that possibly mimic host condition (Lloyd and Allen, 2015). However, in this project bacterial growth rates were investigated for individuals and not in competition with others, which is different to the actual situation but gives useful information for host pathogen interaction experiments.

What is practically important in this work is that in experiments involving host cells, bacteria are inoculated into rich growth medium for specific for period, and rapid growth rate may affect measurements of host pathogen interactions and must be taken into account in any investigation of aspects such as invasion, attachment, and induction of cytokines.

3.1.3 Acid resistance

The pH is a very important factor for pathogens growth, particularly in host environment. The first contact of newborns with pathogenic bacteria is during the delivery process, and handing and feeding the newborns after birth, which leads to establishment of new bioflora on the skin and intestinal tract. The susceptibility of commensal microbiota or pathogenic bacteria to persist in the low pH in neonatal gastrointestinal fluids, is the first step to establish a new relationship with the intestinal mucosa and epithelial cells. The secreted gastric fluid in neonates is enough to generate a fasting gastric pH of 1.5, which increases during feeding to between pH 3.0 and 5.0 (Johnson, 2001; Hill, 2002; Cotter and Hill, 2003; de Jesus *et al,* 2005; Hurrell *et al,* 2009; Alvarez-Ordóñez *et al,* 2014). Moreover, the gastric pH of neonates varied according to the feeding regimes. The gastric pH recorded from neonates fed breast milk only was approximately 2.5, while other regime ranged from 3.5-4.3 (Hurrell *et al,* 2009). However, in some cases of breastfeeding the pH was as low as 1.5, which might have contributed to the lack of biofilm formation by Enterobacteriaceae in enteral feeding tubes obtained from these neonates (Hurrell *et al,* 2009).

To establish infections in neonates, the elderly or other immunocompromised patient, pathogenic agent (e.g., *Cronobacter* spp.) have to persist at very low pH levels of gastric acid. Gorden and Small (1993) stated that *Shigella* and *Escherichia coli* were able to survive at under low acid conditions (pH2.5). However, this can affect the bacterial virulence, as they found that *Shigella* spp lost its ability to invade epithelial cells under these condetions (Gorden and Small 1993).

Acid tolerance is one of the important virulence factor of *Cronobacter* spp that has been investigated. Dancer *et al,* (2009) reported that about 79% of examined *Cronobacter*

strains were able to grow at pH of 3.9. Moreover, Edelson-Mammel *et al,* (2006) Indicated that although growing of *Cronobacter* strains at pH of 3.5 for up to 5 hours reduced the viability by 1 - 3.5 logs, this pH did not kill all of the bacteria.

Alvarez-Ordóñez *et al,* (2014) stated that acid resistance in *Cronobacter* is regulated by different mechanisms including the maintenance of cellular envelope integrity, posttranscriptional modification of tRNA molecules, and repairing the damage in proteins and nucleic acids. Moreover, the envelope stress response regulators OmpR and Cpx are play important roles in *Cronobacter* response to acid stress. (Alvarez-Ordóñez *et al,* 2014). However, it was reported that using organic acids like propionic and acetic acid at pH of 4.0 to 5.0 as antimicrobial factors in liquids like baby food and fruit juice can suppress the growth and survival of *Cronobacter* (Back *et al,* 2009; Al-Holy *et al,* 2010).

The ability of pathogenic bacteria to establish gut infection and cause disease mostly depends on their capability to resist antimicrobial agents produced by the host such as bile and gastric acids (Beceiro *et al,* 2013), therefore, strains were investigated for their resistance to low pH in order to find any link between pathogenicity and acid resistance.

3.1.4 Motility.

Motility, however, is another important virulence factor for pathogenic bacteria, and playing an important role in a bacterial chemotaxis activities and nutrient acquisition (Merrell *et al,* 2003; Sanders *et al.*2013). In *Cronobacter,* flagella are the structures that are responsible for motility, and also play a key role in adherence and invasion to host tissues, triggering host inflammation and help in bacterial cell translocation (Hartmann *et al,* 2010, Amalaradjou and Venkitanarayanan 2011). In addition to flagella, *Cronobacter* may use different traits for motility such as fimbriae (twitching motility) which is another potential virulence factor of this genus. The *Cronobacter* genome contains a number of flageller genes that are *fli* group (*fliR-E*) and *flg* group (*flgL-N*) and twitching motility protein *pilT* (Kucerova *et al,* 2010; Joseph *et al,* 2012a; Choi *et al,* 2014). Hatmann *et al,* (2010) stated that flagella in *Cronobacter* were also involved in different virulence traits such as biofilm formation and adhesion to epithelial cells.

3.1.5 Biofilm formation.

Biofilm production by pathogenic bacteria has been widely investigated. Biofilm formation was associated with virulence in *Escherichia coli* (Naves *et al,* 2008), and *Pseudomonas* (Ghadaksaz *et al,* 2015). Wood *et al,* (2006) indicated that the bacterial motility helps in adhesion to surface by *E. coli,* and the of mechanism regulation of biofilm production and flagella motility in *C. sakazakii,* is also established (Ye *et al,* 2015).

Biofilm formation enhances bacterial resistance to environmental stress and protects bacteria against disinfectants and antibiotics (Furukawa *et al,* 2006), and increases bacterial survival at the very low gastrointestinal pH (Iversen *et al,* 2004; Kim *et al,* 2006; Dancer *et al,* 2009; Jung *et al.*2013). In the food industrial environments, biofilms formation on the food contact surfaces are a main source of contamination with bacteria (Van Houdt and Michiels, 2010).

Moreover, biofilm formation was linked to capsule production by *Cronobacter* isolates. In a study carried out by Iversen *et al,* (2004), the capsulated strains were able to produce detectable amounts of biofilm using infant formula on baby bottles made from various different materials, such as polycarbonate, latex, and silicon, and they suggested that colanic acid capsule might be involved in this process while non-capsulated strains failed to make biofilms. Enteral feeding tubes, which are another possible site for biofilm formation were also investigated. Kim *et al,* (2006) investigated biofilm formation by *Cronobacter* spp on enteral feeding tubes using different medium: infant formula broth (IFB), tryptic soy broth (TSB), and lettuce juice broth (LJB) at 12 and 25°C and concluded that biofilm formation in enteral feeding tubes resulted in bacterial cells aggregation, which may provide the required protection from gastric acid. Motility in *Cronobacter* spp. was associated with increased biofilm and attachment to Caco-2 cells (Hartmann *et al,* 2010).

3.1.6 Capsule production

Capsular polysaccharides (CPS) are major bacterial virulence factors and environmental fitness traits.

Bacterial Capsular polysaccharides (CPS) are major virulence factors that protect bacteria against extreme environmental stress (Shin *et al,* 2012; Ogrodzki and Forsythe, 2015). The structure of bacterial cell surface is one of many factors that contributes bacterial resistance to environmental stress, and represents the first line of defence for a bacterium (Reckseidler-Zenteno 2012). The polysaccharide capsule, which is a common characteristic on bacteria cell wall can play an important role in contributing pathogenicity and resistance to desiccation. Reckseidler-Zenteno., (2012) found that mucoid strains of *Acinetobacter calcoaceticus, E. coli, and Erwinia stewartii* are more resistant to desiccation than non-mucoid isolates. Capsules are composed of different materials including of polysaccharides, polypeptides and polynucleotides (Forsythe 2010; Reckseidler-Zenteno 2012). Colanic acid exopolysaccharide capsules comprises of D-galactose, D-glucuronic acid, D-glucose, L-fucose and pyruvate, which together create a thick mucoid material around the cell (Mao *et al,* 2001).

Caubilla-Barron and Forsythe (2007) demonstrated that capsule producing *Cronobacter* spp isolates were able to persist in dehydrated infant formula for up to forty months, and capsulated strains produced high amounts of biofilms in neonatal gastric tube. (Iversen *et al,* 2004; Hurrell *et al,* 2009a). Danese *et al,* (2000) stated that exopolysaccharides capsule is also involved in the final stages of biofilm formation in *E. coli* K-12.

Conversely, Joseph and Wright (2004) and Schembri *et al,* (2004) reported that capsule production inhibits biofilm formation and ⁄ or bacterial adhesion in *Escherichia coli* K12, *Klebsiella pneumoniae* and *Vibrio vulnificus* (Dancer *et al,* 2009), and this was also confirmed by Hurrell *et al,* (2009b) when non-capsulated *Cronobacter* produced more biofilm than those s mucoid capsules on milk agar medium. Furthermore, the results obtained by Dancer *et al,* (2009) showed that capsule production by *Cronobacter* occurs at conditions with limiting nitrogen sources, meanwhile, biofilm formation was higher under nitrogen-rich conditions, and researchers concluded that capsule is not important for biofilm formation.

Capsules have also been found to protect pathogenic bacteria against host defence reactions like macrophages (Daffe and Etienne, 1999) and bactericidal effects of human serum in *E. coli* (Miajlovic *et al,* 2014).

Capsule production in bacteria is regulated by several genes. McCallum and Whitfield (1991) reported that *rcsA* gene is responsible for production of mucoid capsular material while *wca* operon controls the production of the exopolysaccharide colanic acid which contains fucose and glucuronic acid in *E. coli* K12 (Joseph *et al,* 2012). In *Cronobacter*, the recent study by Ogrodzki and Forsythe (2015) which is based on capsule profiling of genus *Cronobacter* indicated that the capsular polysaccharide K-antigen genes are located in three regions, where region 1 (*kpsEDCS* and 3 (*kpsMT*) are shared by all *Cronobacter* isolates while conservation region 2 varied. Genes located in the region 2 were assigned into two groups (K1 and K2). Moreover, there is a variation in colanic acid (CA) synthesis gene which were categorised into two groups CA1 and CA2 where CA2 is missing the *galE* gene. Moreover, they indicated that Cellulose (*bcs* genes) were absent in *C. sakazakii* strains that belonged to sequence type 13 (ST13) and clonal complex 100 (CC100). These strains considered as (Cell-). Isolates from *C. sakazakii* and *C. malonaticus* with capsule type [K2:CA2:Cell+] were linked to neonatal necrotizing enterocolitis and meningitis (Ogrodzki and Forsythe 2015).

However, 62% of *C. sakazakii* have a K2:CA2:Cell+ capsule profile and 60% of theses isolates are considered clinically significant and were involved in neonatal infections including meningitis, septicaemia or NEC (Ogrodzki and Forsythe 2015).

3.1.7 Haemolytic activity.

Haemolytic activity of some pathogenic bacterial isolates, is an important virulence factor that plays a key role in pathogenicity. Haemolytic activity of specific bacterium is a result of breakdown of red blood cells due to lysing of the cell membrane (Vallet-Gely *et al,* 2010).

This strategy is applied by most bacteria that produce toxins like haemolysin. Previous studies identified three types of haemlysis according to appearance on blood agar plates: β-haemolysing (clear, complete lysis of red cells), α-haemolysing (incomplete, green

hemolysis), and γ-haemolysing (no hemolysis). Alpha haemolysis has been found to be more common in clinical isolates, such as *Serratia* spp and *E. coli*. (Schmidt *et al,* 1995; Balsalobre *et al,* 2006). However, the group B streptococcus sepsis is known to produce β-haemolysin which plays an important role in liver failure and subsequently high mortality rates especially in neonates (Ring *et al,* 2002). However, different isolates from group B streptococcus can manifest α-, β-, or even γ-hemolysis (Patterson, 1996). Joseph *et al,* (2012) found that some *Cronobacter* isolates can produce different haemolysins.

Genetically, haemolysis activity is regulated by several genes. Vallet-Gely *et al,* (2009) found that a mutation in *gac* gene in *Pseudomonas entomophila* resulted in defective in the secretion of haemolysin. Moreover, Joseph *et al,* (2012b) reported that six genes were associated with haemolysis In *Cronobacter*, which are: haemolysin activator protein precursor (ESA_00102), predicted membrane protein haemolysin III homologue (ESA_00432), haemolysins and related proteins containing CBS domains (ESA_00643), haemolysin expression modulating protein (ESA_02810), putative haemolysin (ESA_02937) and a haemolysin precursor (ESA_03540)

The aim of this chapter is to investigate some of phenotypical and physiological traits of selected *Cronobacter* isolates and *E. coli* K1 939 isolate as well as *E. coli* K12 and *Salmonella* Enteritidis as negative and positive controls respectively. This is in order to identify any correlation between these characters and other virulence factors stated in the other chapters such as invasion, attachment, translocation and serum resistance and known *Cronobacter* pathology.

This chapter will include:

- The growth phase using two different medium which are TSB and tissue culture medium (DMEM). This will also allow later evaluation of attachment and invasion studies by taking into account speed of growth in tissue culture medium
- Bacterial sensitivity to 1% v/v Triton X-100 that used to lyse human cells after attachment and invasion assays to avoid killing recovered bacteria by long exposure to triton-X.

- Motility of bacterial isolates on semisolid agar plates
- Phenotypical characters of bacterial colonies according to the binding to Congo red dye
- Acid sensitivity of bacterial isolates mimicking host conditions.
- Biofilm formation using Cow and Gate formula on two different surfaces which are 24-well plate and neonatal enteral feeding tubes.
- Capsule formation on different medium and on different carbon sources
- Haemolysic activity.

3.2 Material and methods.

Materials and methods related to this chapter were described in details in chapter two. However, a brief description is included as needed in results section.

3.3 Results

3.3.1 Growth phase of bacterial isolates.

Growing of microorganisms under conditions of fixed amount of substrate is known as a batch culture, while continuous culture means that the amount of available substrate remains the same during the experimental period (Maier *et al,* 2000), both cultures is used to prepare organisms for range of virulence tests. The growth curve for all bacterial isolates were generated using batch culture method when bacteria were placed in a liquid medium in which the environmental conditions and nutrients are controlled, this method is typically use to understand and define the growth of a particular microbial isolate. The growth curve was gathered for all of bacterial isolates in two different types of medium (TSB and DMEM tissue culture medium) to determine whether there were any differences that may effect on their general physiological features.

3.3.1.2 Growth phase in TSB.

Twenty-nine strains were investigated in this experiment including *E. coli* K12 (1230) and *Salmonella* enteritidis (358) as negative and positive controls respectively in experiments studying host pathogen interactions. Bacterial isolates were prepared as described in section (2.4), briefly; from agar plates 1-2 colonies added to 5 ml of TSB and incubated for 16-24h at 37^{o}C and shaking. Then bacterial Isolates adjusted to starting optical density (OD_{600}) of 0.05 in fresh TSB medium and incubated at shaker at 200 rpm and 37^{o}C. Bacterial growth was then determined using OD data recorded for triplicates at 600nm of eight different time points (0 immediumtely after diluted, 3, 6, 10, 20, 24, 28 and 32 hrs), in order to determine the growth curve of the tested bacterial strains. The OD of bacterial culture was measured using plate reader (BioTek, UK). Results shown in figures 3-1, 3-2 and 3-3 indicated that all strains showed an exponential increase during the first 10 hours especially in the earliest first hours of incubation, with recorded increase up to 8-fold of the initial value.

However, this finding suggests that strains from overnight culture (16-24h) used in this project were at stationary phase.

Although, some strains showed a minor increase in the OD between 10 and 28 hrs incubation, it can be considered that the stationary growth phase started at some point around 10 hours for all strains. Growth varied from one strain to another, and the optical density ranged from 0.24 to 0.45 at the beginning of stationary phase. However, *C. dublinensis* strain 1556 showed highest growth among of all *Cronobacter* isolates with about 8 folds of the initial value (Figure 3-3), followed by *C. sakazakii isolates* 984 and 693 (Figure 3-2). Conversely, *C. muytjensii* strain 1561 (Figure 3-1) and *Salmonella* Enteritidis (Figure 3-3) were the lowest OD readings at most time points.

Comparatively, *E. coli* K1 strain 939 (Figure 3-3) revealed the highest growth with OD_{600} of 0.45 after 10 hrs incubation, which is about 9 folds of the starting inoculum compared with five folds for *Salmonella* enteritidis the positive control used in most experiments (OD 0.25).

A gradual decrease in culture turbidity was observed by some strains after 24 hours e. g *C. sakazakii strains* 696, 701, 1249, 313 and 1557, *C. universalis* 581, C. *dublinensis* 1556

and *C. malonaticus* strains 681, 1558 and 1569 while the rest of strains maintained four hours of stationary phase until 28h. Overall, most strains revealed a similar pattern on their growth curves.

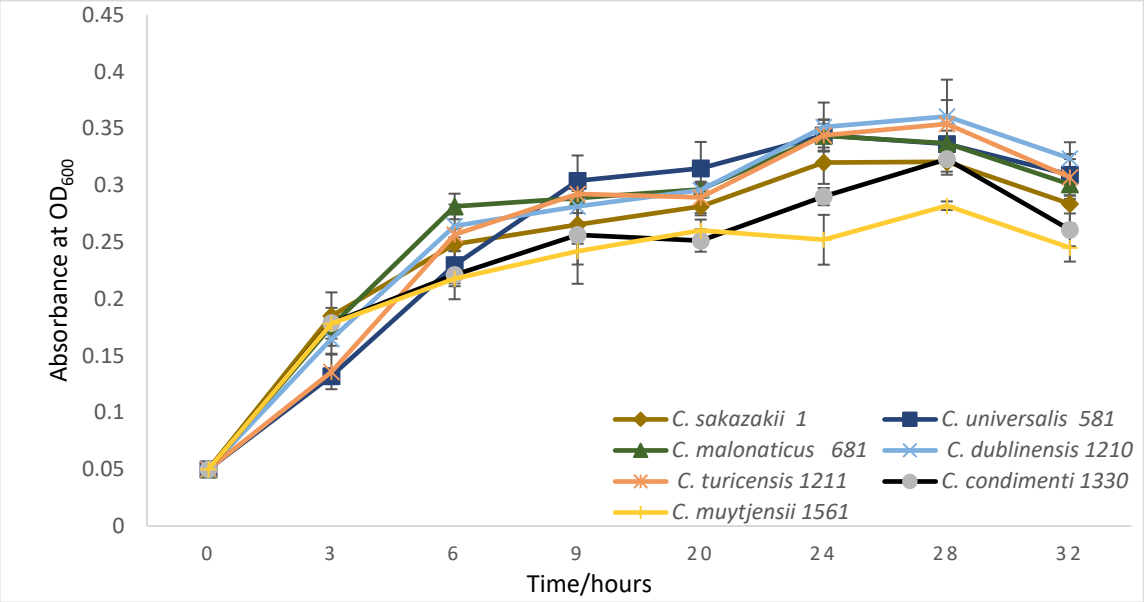


Figure 3-1 Growth curve of 7 *Cronobacter* type species strains in broth medium. Strains were incubated in TSB medium at 37°C for 32 hours, and optical density measured at 8 time pointes. Data shown is the mean of three replicates with error bars showing standard deviation.

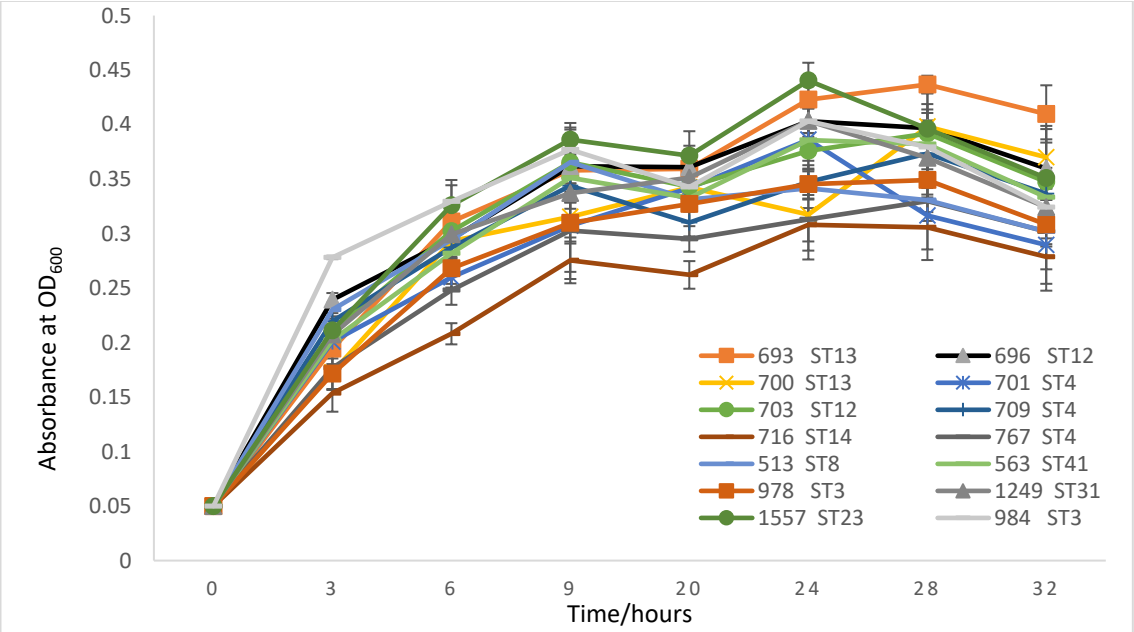


Figure 3-2 Growth curve of selected *Cronobacter sakazakii* isolates in broth medium. Strains were incubated in TSB medium at 37°C for 32 hours, and optical density measured at 8 time points. Data shown is the meanof three replicates with error bars showing standard deviation.

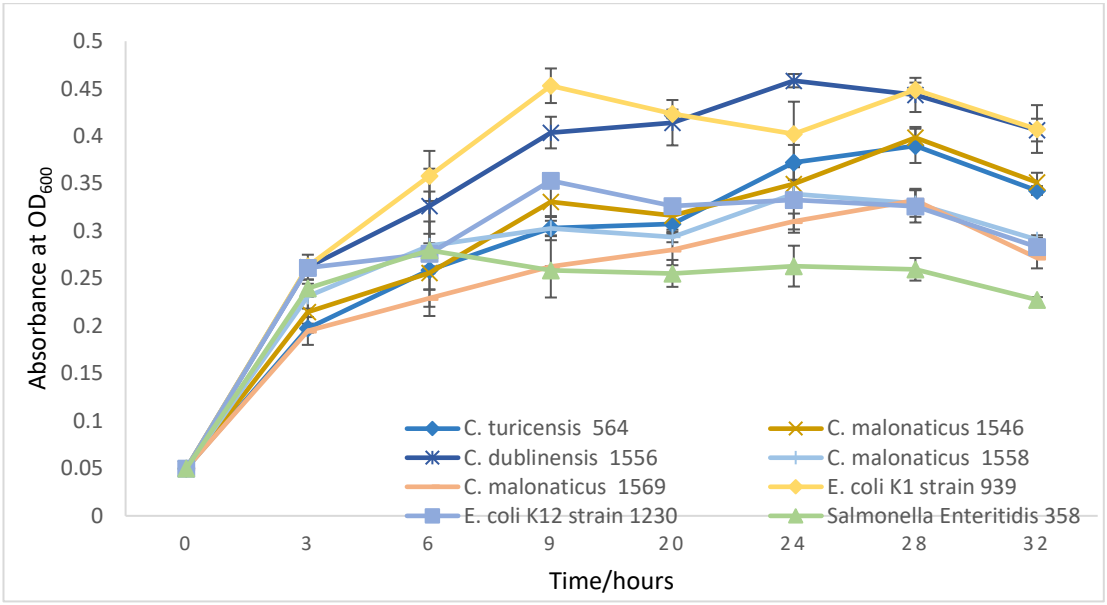


Figure 3-3 Growth curve of selected *bacterial* isolates in broth medium. Strains were incubated in TSB medium at 37°C for 32 hours, and optical density measured at 8 time point. Data shown is the mean of three replicates with error bars showing standard deviation.

3.3.1.3 Growth rate in Tissue culture medium.

Tissue culture experiments involve inoculating the bacteria into rich growth medium to determine their interaction with human cells. Whilst care is taken to inoculate bacteria at the same concentration, it is important to determine whether some strains have increased in numbers more rapidly than others over the typical 3h incubation of the experiment.

Bacterial strains were investigated with regard to their growth in tissue culture medium. As many protocols state an initial concentration of bacteria added to host cells, determining how this inoculum alters during the typical 3h duration of an experiment is important. Results show the change in optical density over 3h in antibiotic-free tissue culture medium and a startinginoculum of OD_{600} 0.05 (figures 3.4, 5, and 6).

The highest OD among *Cronobacter* type species revealed was by *C. malonaticus* strain 681 as it can be clearly seen in Figure 3-4, followed by *C. muytjensis* strain 1561 and *C. turicensis* strains 1211 which showed approximately similar optical density at most of the time points. However, *C. dublinensis* type species strain 1210 and *C. condimenti* type species strain1330 demonstrated lower optical densities which were about threefold of the inoculum compared with fivefold for strain 681. *C. sakazakii type* species strain 1

and *C. universalis* type species 581 both showed medium level of growth rate which is about four folds of the strating density. Comparatively, *E. coli* K1 isolate 939 showed the greatest growth throughout (OD_{600} = 0.3) followed by *C. malonaticus* isolate 681 (Figure 3.4). Such alteration in inoculum during incubationshould be taken into account when examining tissue culture cell-pathogen interactions.

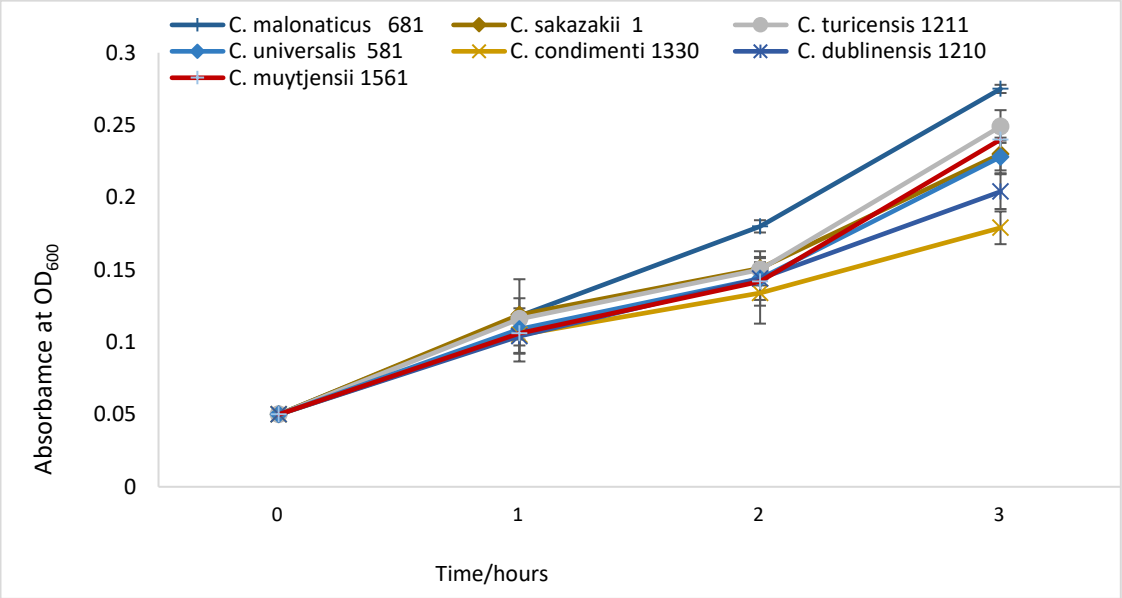


Figure 3-4. Growth curve of 7 *Cronobacter* type species and *E.coli* K1 939 in tissue culture medium. Strains from 18-24h liquid culture were adjusted to OD_{600} of 0.05 and incubated for 3h at 5% Co_2 and 37°C to mimic experimental conditions in adhesion and invasion experiments. Data shown is the mean of three replicates with error bars showing standard deviation.

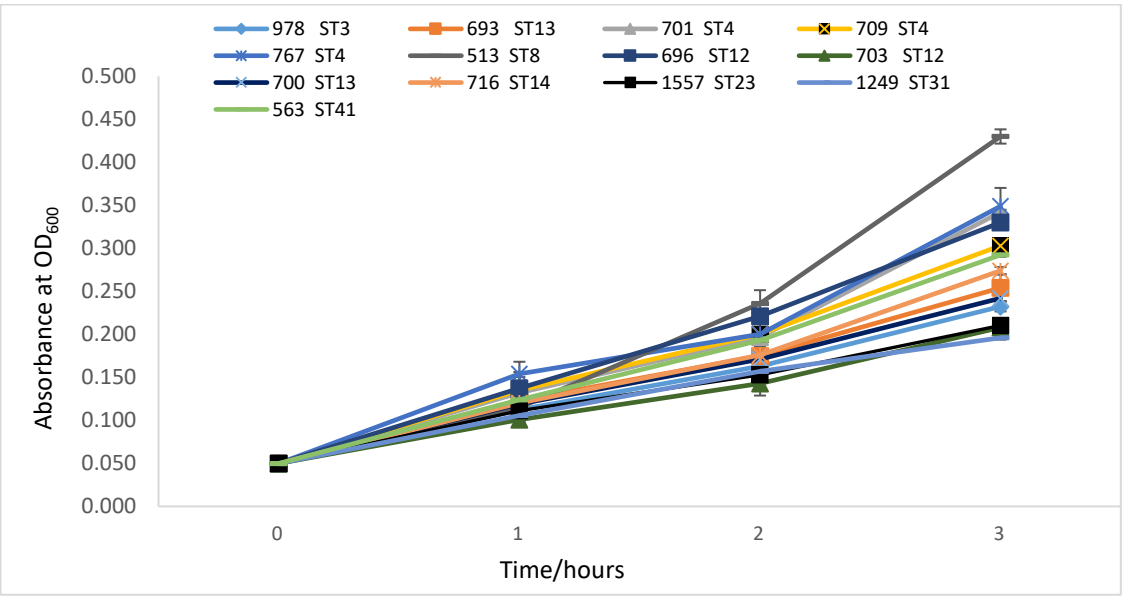


Figure 3-5. Growth curve of selected *C. sakazakii* isolates in tissue culture medium. Strains from overnight culture were adjusted to OD_{600} of 0.05 and incubated for 3h at 5% Co_2 and 37°C to mimic experimental condition in adhesion and invasion experiments. Data shown is the mean of three replicates with error bars showing standard deviation.

Growth curves of *Cronobacter sakazakii* isolates from different STs are shown in figure 3.5. Interestingly strains linked with serious pathology especially in neonates displayed the highest optical density. *C. sakazakii* ST8 strain 513 showed about 9-fold increase compared with the inoculum after 3 hours. *C. sakazakii* ST4 strains 767, 701, and *C. sakazakii* ST12 strain 696, all of them showed about 7f-old increase compared with the inoculum. Strains 767 and 701 were isolated from trachea and peritoneal fluid of fatal meningitis cases and NEC III of two different neonates respectively in French 1994 outbreak. Both strains were from pulsed-field gel electrophoresis (PFGE) profile 2 which is a possibly suggests that the two isolates are the same strain. Though, *Cronobacter* sakazakii ST23 (1557), ST31 (1249) and *C. sakazakii* ST12 (703) showed lowest growth among other STs (4-fold increase compared with the inoculum).

Growth of other *C. sakazakii* isolates ranged between 4 and 6-fold increase compared with the inoculum, and most strains showed same mode of growth with a gradualy increase in the optical density, while the OD was sharply increased for some at time point 4 (3h), particularly, *C. sakazakii* strains 513 and 767.

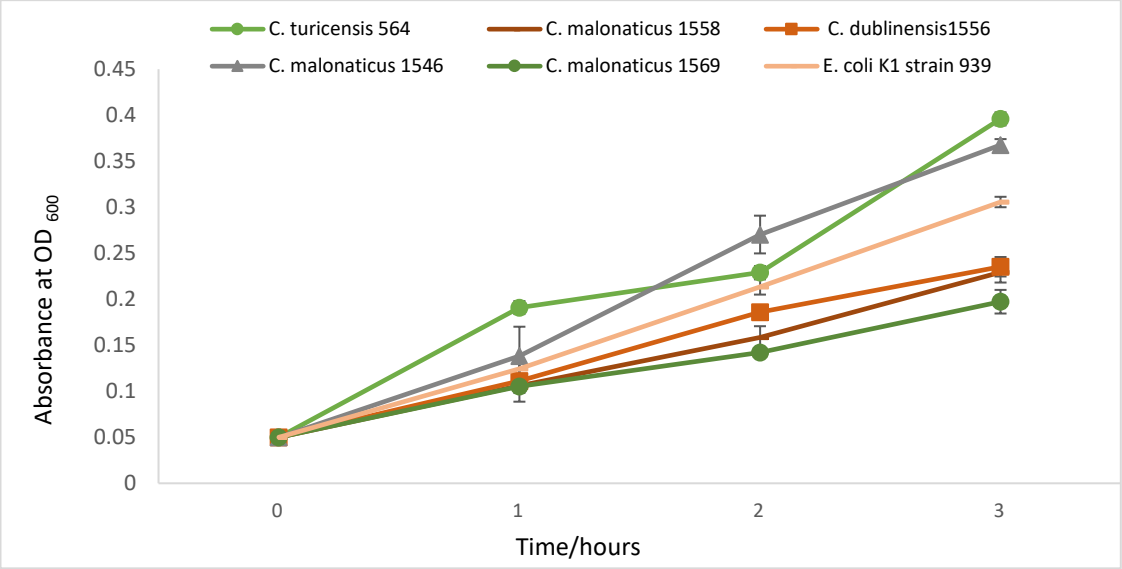


Figure 3-6 Growth curve of selected *Cronobacter* isolates in tissue culture medium. Strains from overnight culture were adjusted to OD_{600} of 0.05 and incubated for 3h at 5% CO_2 and 37°C to mimic experimental condition in adhesion and invasion experiments. Data shown is the mean of three replicates with error bars showing standard deviation.

The results of the other *Cronobacter* isolates were more varied and the increase of the OD ranged from four to eight folds of the inoculum. *C. turicensis* and *C. malonaticus isolates* 564, 1546 respectively was the highest and displayed seven and eightfold

increase compared with the inoculum, while the rest of isolates ranged between four and fivefold increase compared with the inoculum (Figure 3-6). Generally, all strains showed a similar growth phase to that with TSB medium.

One of the most important findings in this experiment was that although all strains started at the same cell density, there was a major difference between their growth rates noted in both medium, which was evident over the first three hours of incubation the period that for all of host-pathogen interaction studies in this project.

3.3.2 Low acid sensitivity assay.

Previous studies showed that some *Cronobacter* isolates displayed an obvious tolerance towards a pH of 3.5 after 2 hours incubation in formula based environment (Hana Sonbol 2015, unpublished data). It has been shown that the gastric pH at birth usually ranges between 6 and 8, and rapidly drops within the first 3 to 5 hours to adult values which are approximately pH 1–5 (Hurrell *et al,* 2009a; Tayman *et al,* 2011)

This experiment was designed to investigate the sensitivity of *Cronobacter* isolates and other comparative strains to very low pH (≤ 2.0) in an environment where no potential protection factors were available, as it was determined that some bacterial isolates revealed different levels of acid tolerance based on the formula type used in the assay (personal communication with Dr. Michael Loughlin). In brief, 18-24h cultures were prepared as indicated in section 2.4. Afterwards, bacteria were incubated in TSB medium at two different pH (2.0 ± .02 and 7.2 ± .02) in triplicates at initial inoculum of OD_{600} of 0.05 for 2 hrs, which is a minimum recommended time between newborns feed (8-12 times/24 hours) (Boskabadi *et al,* 2010) which is also the optimum transition time of the food through stomach. Following the incubation, 200µl from each culture were transferred into a new 96-well plate, and serially diluted in Fresh TSB to log 10^{-11}, and incubated fro 24h at 37^{o}C. Bacterial sensitivity to this low pH was observed by serially diluting of the tested bacteria in fresh TSB in 96-wells and incubated for 24h, then results was obtained as a log cycle and compared with the standard wells that incubated at normal TSB pH (≈7 .2). Results indicated that all of *Cronobacter* isolates were sensitive to low pH and no growth was recovered for all investigated strains after 24h incubation

in TSB medium. Interestingly, both negative control *E. coli* K12, positive control *Salmonella* Enteritidis and *E. coli* K1 strain 939 showed different levels of growth after 24h, which were 4, 6 and 4 logs respectively. For standard controls, all investigated strains grown in normal TSB for the 2 hrs, and then serially diluted in fresh medium, and showed growth of 7-8 logs after 24h.

3.3.2.2 Genes associated with acid tolerance.

Strains were searched for the presence of *ompR* gene which is potentially responsible for the acid tolerance in *Cronobacter* using BLAST *Cronobacter* MLST database (https://pubmlst.org/*cronobacter*/). Results showed that all of strains possess this particular gene, which may explain the ability of this genus to grow at the pH3 and above.

To confirm the negative results obtained from low pH assay and the disability of strains to grow or survive at this pH, they were further screened for acid resistance related genes that give *E. coli* ability to grow at this level (pH 2 ± 0.2). Masuda and Church, 2003 reported that the overexpression of the response regulator *evgA* gene can confer a phenotypic resistance to low acid environment, and the deletion of any of *ydeP*, *yhiE* or *ydeO* operons can cause loss of this virulence. However, *Cronobacter* isolates were negative for yhiE and *ydeO*, while ydeP was present in all strains except in *C. sakazakii* 1557. Also, BLAST search using *Cronobacter* MLST data base indicated that all *Cronobacter* isolates were negative for acid resistance related genes (*gadA, gadB and gadC* and *hdeA* and *hdeB*) that were described by Masuda and Church, (2002*)* in *E. coli*. This analysis was performed in order to find any similarity between these two species, which might explain the ability of *E. coli* to survive low pH, while *Cronobacter* do not. However, although the *RpoS* (RNA polymerase sigma) which regulating the acid, heat and salt tolerance (Cheville *et al,* 1996) was positive in all of tested *Cronobacter* strains, they showed high sensitivity to pH 2 and no growth was observed, which might regulated by different mechanisms.

3.3.3 Bacterial sensitivity to 1% Triton X-100

Schnaitman. (1971) stated that Triton X-100 have an effect on the cell wall protein aggregation and membrane permeability, which might have a further effect on the bacterial viability. This reagent is used in tissue culture to lyse epithelial cells in attachment and invasion experiment for maximum time of 5-10 minutes, and then diluted in PBS. Thus, bacterial isolates were tested for their sensitivity to a concentration of 1% v/v Triton-X 100 similar to that used in tissue culture experiment. After incubation with Triton-X 100 as described in section 2.8.4.4, bacteria were then recovered by serially dilution in TSB medium using a 96-well plate and incubated at 37^{o}C overnight. Similarly, serial dilution from same overnight culture without Triton-X 100 of each strain was made in TSB to be used as a comparative control with Triton-X results, in order to check if there is any reduction in bacterial number (log) after treatment. However, all strains were resistance to 1% v/v Triton-X 100 with maximum reduction of one log in bacterial numbers after one hour treatment, which is much more than the time incubated in the process of recovering the attached/invaded bacteria into human cell in tissue culture experiments.

3.3.4 Phenotypical characteristics and colony morphology types of bacterial isolates.

3.3.4.2 Morphological appearance of bacterial isolates on different medium

Bacterial colony morphology is a description of the visible characteristics of a pure growing colony. Although, the morphological differences are not a reliable way to identify bacteria, and different types of bacteria might have similar colony appearance, it is very important for initial presumptive identification of bacterial isolates. Colonies that vary in their morphological appearance brought about by phenotypic factors can be different bacterial strains, species, or even genera**:** (Sousaet *et al,* 2013). Thus, bacterial isolates were investigated for their morphological characteristics on six different medium (TSA, XLD, VRBGA, VRBLA, DFI and MacConkey agar medium) see section (2.8.4.6.1).

Colony appearance and texture were varied. Strains morphology was approximately similar on the same medium with minor differences, especially on VRBGA, VRBLA medium where colours were either mucoid light pink to dark purple. However, on MacConkey agar medium, some strains were not able to produce any mucoid material and appeared dry. On TSA medium, all strains were yellow to creamy and the texture differed from light mucoid to leathery and sticky as shown by *C. malonaticus* (681) and *C. sakazakii* isolates 690 and 717, while for *C. sakazakii* 718 the growth was thick and granulated. Most of the strains were yellow and mucoid on XLD medium except *C. malonaticus* 1569, and *C. sakazakii* isolate 1, 696 and 716. Strains were also grown on Druggan Forsythe Iversen (DFI) agar medium, which is a selective medium for *Cronobacter* (Iversen *et al,* 2004). Most strains give blue colour and none of them were mucoid except *C. sakazakii isolates* strains: 1, 717, 1249 and 1557 and *C. malonaticus* isolates: 1546, 1558 and 1569 and *C. dublinensis isolate 1556.* All results can be seen in table 3-1.

Table 3-1. Morphological characteristics of bacterial isolates on different agar medium.

Strain Information			TSA	DFI	VRBGA	VRBLA	XLD	MacConkey
Isolate	Species	ST						
978		3	Y-Cr	Blue/D	P-Pur/M	Pur/M	Y/M	Pur/M
984		3	Y-Cr	Blue/D	Pur/M	Pur/M	Y/M	Pur/M
712		4	Y-Cr/M	Blue/D	P/M	Pur/M	Y/M	P/M
767		4	Y-Cr/M	Blue/D	P/M	P-Pur/M	Y/M	P/M
691		4	Y-Cr/M	Blue/D	P/M	P/D	Y/M	P/M
692		4	Y-Cr/M	Blue/D	P/M	Pur/M	Y/M	Pur/M
694		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
695		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
698		4	Y-Cr/M	Blue/D	P-Pur/M	P-Pur/M	Y/M	P/M
701		4	Y-Cr/M	Blue/D	P/M	P-Pur/M	Y/D	P/M
702		4	Y-Cr/M	Blue/D	P/M	P-Pur/M	Y/M	P/M
705		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
706		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
707		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
709		4	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
711		4	Y-Cr/M	Blue/D	P/M	Pur/M	Y/M	P/M
730		4	Y-Cr	Blue/D	P/M	P/M	Y/M	P/D
1	*C. Sakazakii*	8	Y-Cr	Blue-G/D	P/M	P/M	Y/M	P/D
513		8	Y-Cr	Blue/D	P-Pur/M	Pur/M	Y/M	Pur/M
690		12	Y-Cr/Le& Sticky	Blue/D	P-Pur/M	Pur/M	Y/M	Pur/D
696		12	Y-Cr/M	Blue/D	P/M	P/M	Y/D	P/M
699		12	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
708		12	Y-Cr/M	Blue/D	P/M	P-Pur/M	Y/M	P/M
703		12	Y-Cr/M	Blue/D	P/M	Pur/M	Y/M	P/M
693		13	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
700		13	Y-Cr/M	Blue/D	P/M	P-Pur/M	Y/M	P/M
713		13	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
714		13	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
715		13	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/M
716		14	Y-Cr/M	Blue/D	P/M	P/M	Y/M	P/D
717		14	Y-Cr/Le& Sticky	Blue/D	P/M	P/M	Y/M	P/D
718		14	Y-Cr/thi& granu	Blue/D	P/M	P/M	Y/M	P/D
1557		23	Y-Cr/Thick-M	G-B/D	P-Pur/M	Pur/M	Y/M	P/D
1249		31	Y-Cr	G-B/D	P-Pur/M	Pur/M	Y/M	Pur/M
563		41	Y-Cr	Blue/D	P/M	Pur/M	Y/M	Pur/D

Druggan-Forsythe-Iversen (DFI) medium, Tryptic Soy Agar (TSA), Violet Red Bile Glucose Agar (VRBGA) medium, Violet Red Bile Lactose Agar medium (VRBLA) PHENOTYPING, Xylose lysine Deoxycholate agar (XLD) medium and MacConkey Agar. **Y-Cr/M**: yellowish creamy and mucoid; Y**-Cr/Le& Stick**: yellowish creamy and leathery-stick in texture; **Y-Cr/thi&granu**: yellowish creamy very thick and granulated in texture; **Blue/D**: blue and dry; **G-B/D**: greenish blue and dry; **Pur/M**: purple and mucoid; **P/M**: pink and mucoid; P**-Pur/M**: Pink to purple and mucoid; **P/D**: Pink and dry; **Pur/D**: purple and dry; **Y/M**: yellow and mucoid**; Y/D**: yellow and dry.

Table 3-1 continued.

Strain Information			TSA	DFI	VRBGA	VRBLA	XLD	MacConkey
Isolate	Species	ST						
681		**7**	Y-CR/ lea&sticky	Blue/D	P-Pur/M	P-Pur/M	Y/M	P/D
1558	*C. malonaticus*	**7**	Y-CR	G-B/D	P-Pur/M	PUR/M	Y/M	P/D
1546		**84**	Y-CR	G-B/D	P/M	PUR/M	Y/M	Pur/D
1569		**307**	Y-CR	G-B/D	P-Pur/M	PUR/M	Y/D	Pur/D
564	*C. turicensis*	**5**	Y-CR	Blue/D	P/M	PUR/M	Y/M	Pur/M
1211		**19**	Y-CR	Blue/D	P-Pur/M	PUR/M	Y/M	Pur/M
1556	*C. dublinensis*	**80**	Y-CR	G-B/D	P-Pur/M	PUR/M	Y/M	Pur/M
1210		**106**	Y-CR	Blue/D	P-Pur/M	PURPLE	Y/M	P/M
581	*C. universalis*	**54**	Y-CR	Blue/D	PUR/D	P/M	Y/M	P/D
1330	*C. condimenti*	**98**	Y-CR	Blue/D	P-Pur/M	PUR/M	Y/M	Pur/M
1561	*C. muytjensii*	**81**	Y-CR	Blue/D	PUR/D	P/M	Y/M	Pur/M

Druggan-Forsythe-Iversen (**DFI**) medium, Tryptic Soy Agar (**TSA**), Violet Red Bile Glucose Agar (**VRBGA**) medium, Violet Red Bile Lactose Agar medium (**VRBLA**), Xylose lysine Deoxycholate agar (**XLD**) medium and MacConkey Agar. **Y-Cr/M**: yellowish creamy and mucoid; **Y-Cr/Le& Stick**: yellowish creamy and leathery-stick in texture; **Y-Cr/thi&granu**: yellowish creamy very thick and granulated in texture; **Blue/D**: blue and dry; **G-B/D**: greenish blue and dry; **Pur/M**: purple and mucoid; **P/M**: pink and mucoid; **P-Pur/M**: Pink to purple and mucoid; **P/D**: Pink and dry; **Pur/D**: purple and dry; **Y/M**: yellow and mucoid**; Y/D**: yellow and dry.

3.3.4.3 Congo red phenotypic assay.

The colony morphology of *Cronobacter* isolates on Congo red agar medium was studied for the binding of the Congo red dye to bacterial isolates at two different temperatures. Colony morphology was categorised as described by Yan *et al,* (2015) into four main groups which are: red and smooth (RAS); red, dry, and rough (RDAR); brown and smooth (BAS) and brown, dry, and rough (BDAR). Examples shown in figure 3-7.

Accordingly, the most common morphotype among *Cronobacter* isolates at both investigated degrees (28^{o}C and 37^{o}C that matching skin and body temperature respectively) was BAS (34/46) at 28^{o}C after 72hrs and (23/46) at 37^{o}C after 48hrs. The changes in colony morphology was followed for 48 hours for strains incubated at 37^{o}C and 72 hrsm. For those incubated ta 28^{o}C colonies were exameied evey 24h, to allow strains that showed weak Congo red binding to develop the desired morphotype. At

37°C, some strains revealed a changes of the colour seen after 24h of incubation. For example: *C. sakazakii* ST3 strain 978 the colour shifted from BDAR at 24h to RDAR after 48hrs incubation but not for the 984 from same ST group. This changes were also noted for *C. muytjensii* strain 1561 which changed from RAS to BAS, *C. turicensis* isolate 564 from RDAR to BDAR and 1557 BDAR to BAS (Table 3-2). Interestingly, some strains that are thought to be responsible for serious disorders in neonates revealed RDAR colonies of both temperatures, suggesting the ability to express curli fimbriae in body and skin temperature, with possibility to produce biofilm.

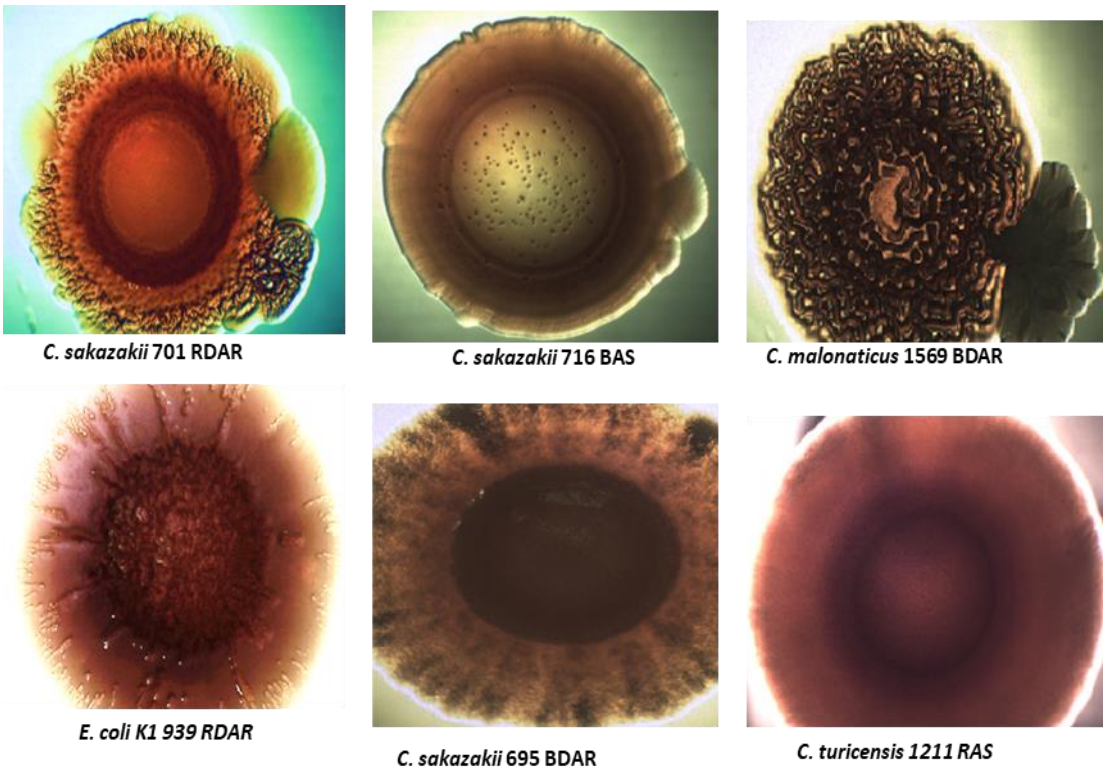


Figure 3-7 Colony morphology of bacterial isolates on Congo red agar plates after 24hr at 37°C. Differences in colony morphology showing different phenotypes of bacterial binding to the dye. **RAS**: red and smooth; **RDAR**: red, dry, and rough; **BAS**: brown and smooth; **BDAR**: brown, dry, and rough. Description is adapted from Yan *et al,* (2015).

Table 3-2. Appearance of *Cronobacter* isolate colony on Congo red medium.

STRAIN INFORMATION				Temperature and duration of incubation				
Isolate	Species	Disease	ST	37°C 24hrs	37°C 48hrs	28°C 24hrs	28°C 48hrs	28°C 72hrs
978		Enteral Feeding Tube	**3**	BDAR	RDAR	BAS	BAS	BAS
984		Enteral Feeding Tube	**3**	BDAR	BDAR	BAS	BAS	BAS
712		Non clinical	**4**	BAS	BAS	BAS	BAS	BAS
767		Fatal infant meningitis	**4**	BAS	BAS	BAS	BAS	BAS
691		ND	**4**	BAS	BAS	BAS	BAS	BAS
692		NECII	**4**	BAS	BAS	BAS	BAS	BAS
694		NECII	**4**	BAS	BAS	BAS	BAS	BAS
695		NECII	**4**	BAS	BAS	RDAR	RDAR	BDAR
698		NECII	**4**	BAS	BAS	BAS	BAS	BAS
701		NECIII	**4**	RDAR	RDAR	RDAR	RDAR	RDAR
702		NECI	**4**	BAS	BAS	BAS	BAS	BAS
705		NECII	**4**	BAS	BAS	BAS	BAS	BAS
706		NECII	**4**	BAS	BAS	BAS	BAS	BAS
707		NECII	**4**	BAS	BAS	BAS	BAS	BAS
709		Septicaemia	**4**	BAS	BAS	BAS	BAS	BAS
711		Asymptomatic	**4**	RAS	RAS	BAS	BAS	BAS
730		NECI	**4**	RAS	RAS	BAS	BAS	BAS
1	***C. sakazakii***	Child's Throat isolate	**8**	RAS	RAS	RDAR	RDAR	RDAR
513		Clinical (ND)	**8**	RDAR	RDAR	RAS	RAS	RAS
690		Asymptomatic	**12**	RDAR	RDAR	RDAR	RDAR	RDAR
696		NECII	**12**	RAS	RAS	BAS	BAS	BAS
699		ND	**12**	BAS	BAS	RDAR	RDAR	BDAR
708		Asymptomatic	**12**	RAS	RAS	BAS	BAS	BAS
703		NECII	**12**	RAS	RAS	BAS	BAS	BAS
693		Asymptomatic	**13**	BAS	BAS	BAS	PAS	RAS
700		Digestive Problem	**13**	BAS	BAS	BAS	BAS	BAS
713		Non clinical	**13**	BDAR	BDAR	BAS	BAS	BAS
714		Non clinical	**13**	BDAR	BDAR	BAS	BAS	BAS
715		Non clinical	**13**	BDAR	BDAR	BAS	BAS	BAS
716		Non clinical	**14**	RDAR	RDAR	BAS	BAS	RAS
717		Non clinical	**14**	RDAR	RDAR	RDAR	RDAR	RDAR
718		Non clinical	**14**	RAS	RAS	RDAR	RDAR	RDAR
1557		Bronchial secretion*	**23**	BDAR	BAS	RDAR	RDAR	RDAR
1249		Fatal infant meningitis	**31**	BAS	BAS	BAS	BAS	BAS
563		Foot wound	**41**	RDAR	RDAR	BAS	BAS	BAS

RAS: red and smooth; **RDAR**: red, dry, and rough; **BAS**: brown and smooth; **BDAR**: brown, dry, and rough; **ND**: no details; **ST**: sequence type. NECI, II, III: necrotising enterocolitis stage 1, 2 and 3.

Table 3-2 continued

Strain information				Temperature and duration of incubation				
Isolate	Species	disease	ST	37°C 24hrs	37°C 48hrs	28°C 24hrs	28°C 48hrs	28°C 72hrs
681	*C. malonaticus*	Clinical (ND)	**7**	BAS	BAS	RDAR	RDAR	RDAR
1558		Clinical (ND)	**7**	RDAR	RDAR	BAS	BAS	BAS
1546		Clinical (ND)	**84**	RAS	RAS	BAS	BAS	BAS
1569		Fatal infant meningitis	**307**	RDAR	RDAR	RDAR	RDAR	RDAR
564	*C. turicensis*	Neonatal Meningitis	**5**	RDAR	BDAR	PAS	PAS	PAS
1211		Fatal infant infection	**19**	BAS	BAS	BAS	PAS	RAS
1556	*C. dublinensis*	Abscess	**80**	BAS	BAS	BAS	BAS	BAS
1210		Non clinical	**106**	BAS	BAS	BAS	PAS	RAS
581*	*C. universalis*	Non clinical	**54**	RAS	RAS	BAS	BAS	BAS
1330	*C. condimenti*	Non clinical	**98**	BAS	BAS	BAS	PAS	RAS
1561	*C. muytjensii*	Non clinical	**81**	RAS	BAS	BAS	PAS	RAS

RAS: red and smooth; **RDAR**: red, dry, and rough; **BAS**: brown and smooth; **BDAR**: brown, dry, and rough; **ND**: no details; **ST**: sequence type.

Binding of bacterial cells to Congo red dye was previously used as indicator to the possessing of bacterial cells to curli fimbriae. Strains that showed more binding to Congo red dye revealed greater motility and biofilm production (Finn *et al,* 2013; Yan *et al,* 2015). Taking into consideration the finding of Daskaleros and Payne (1987) that binding to Congo red might indicate to the ability of the bacteria to bind to blood hemin and utilize Iron from host blood, which is an important virulence. According to Yan *et al,* (2015) isolates with morphotypes RDAR or BDAR were considered positive for the binding to Congo red dye. In this assay, 34% of the tested isolates incubated at 37°C and 23% of the same isolates when incubated at 28°C. 66% of isolates showed either RDAR or BDAR were clinical isolates or isolated from clinical sites, including five strains isolated from Infant formula in France 1994 outbreak.

3.3.5 Motility assay of bacterial isolates.

Forty nine strains were investigated including negative and positive controls *E.coli* K12 and *Salmonella* Enteritidis respectively and one *E. coli* K1 (939) as a comparative isolate

from a different genus. Motility assay was performed on semisolid agar plates supplemented with Triphenyl- tetrazolium chloride (TTCS) dye.

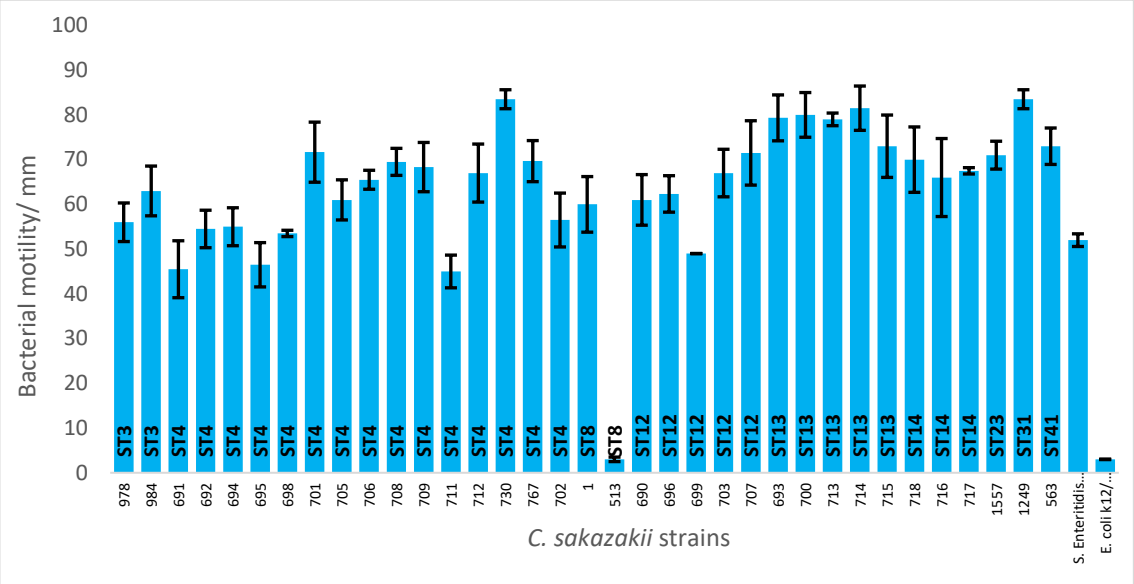


Figure 3-8 Motility of selected *C. sakazakii* isolates on semisolid medium containing TTCS dye. *Salmonella* Enteritidis and *E. coli* K12 used in tissue culture experiment as a positive and negative controls respectively indicated by stars. Three microliters of overnight culture were inoculate in the middle of the plate and incubated 18h at 37°C. Data shown as a mean of three independent experiments with error bars showing standard deviation. Numbers inside or above the columns indicate the sequence type (ST, i.e. ST4, ST8 etc.)

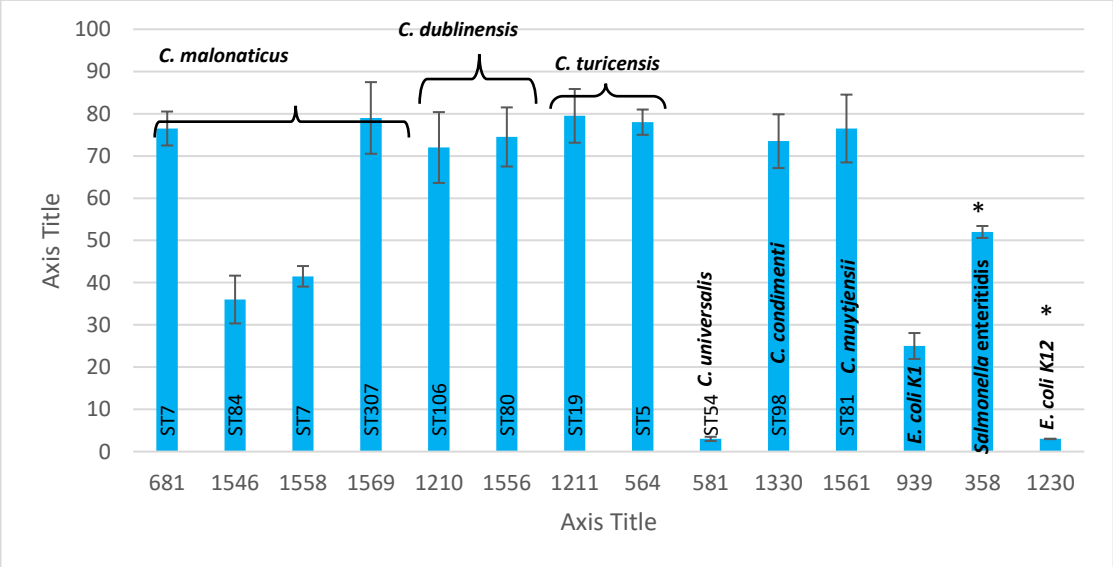


Figure 3-9 Motility of selected *Cronobacter* isolates and *E. coli* K1 939 on semisolid medium. *Salmonella* Enteritidis and *E. coli* K12 used in tissue culture experiment as a positive and negative controls respectively indicated by stars. Three microliter of overnight culture were inoculate in the middle of the plate and incubated 18h at 37°C. Data shown as a mean of three independent experiments with error bars showing standard deviation. **Numbers** inside or above the columns indicate the sequence type (ST, i.e. ST4, ST8 etc.)

Bacterial motility was varied, and strains showed substantial differences in the motility zone especially within the ST8 group, where one of the two isolates was non-motile (513) and strain1 showed 60 mm motility zone. Motility zone *C. sakazakii* ST3 isolates ranged from 56 to 63 mm, while ST4 isolates showed more variation and ranged from 45 mm to 83 mm (figure 3.8). ST12 isolates displayed motility zones of about 50-71mm, and there was no variation between ST13 isolates that showed almost the highest motility. Motility zone other *C. sakazakii* strains ranged from 66-83mm. Examples shown in figure 3-10.

Moreover, high variation was shown for *C. malonaticus* isolates and strain 1569 ST112 was the highest motile (figure 3.9). *C. turicensis* isolates 564 and 1211 produced zone of motility ranging 78-80mm. The two *C. malonaticus* strains 1546 ST84 and 1558 ST89 produced zone of motility 36 mm and 41.5 mm respectively (Figure 3.9). However, *C. universalis* strains 581 (NTU type strain) was also completely non-motile.

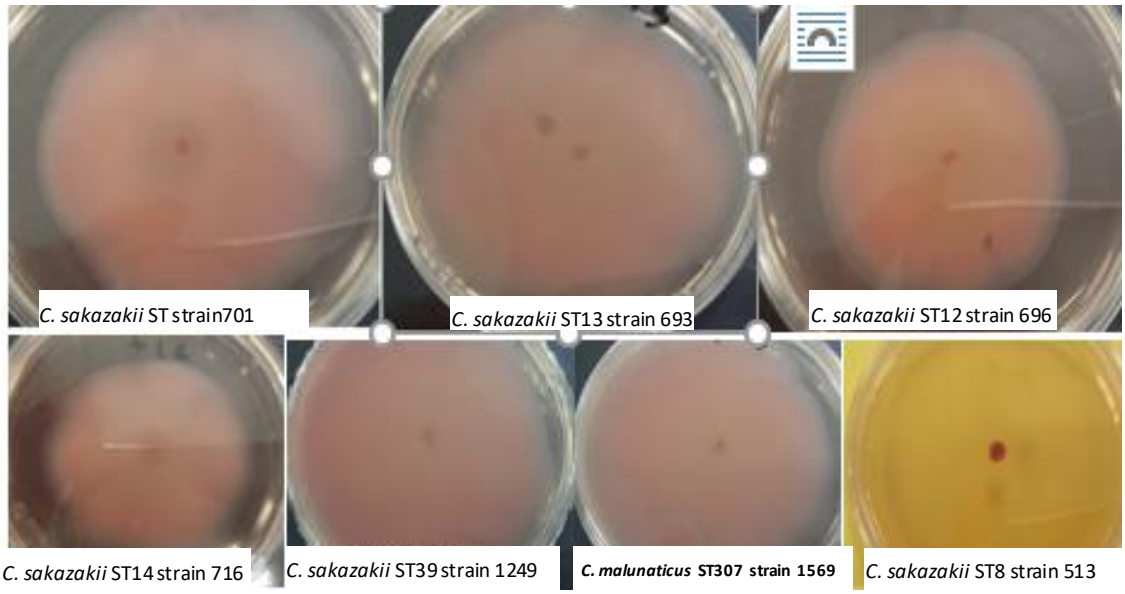


Figure 3-10 Motility of selected bacterial isolates through semi solid medium. Three microliters of 18-24h broth culture were inoculated in the middle of the plate and incubated 24h at 37°C. 0.5 ml of Triphenyl- tetrazolium chloride, solution (TTCS) was added to100 ml medium (v/v) before sterilization to visualize the bacterial growth and motility.

3.3.5.2 Genes responsible for motility.

Cronobacter strains were screened for *fli* (ESA_01248–61) and *flg* (ESA_02264–77) genes using BLAST option of the online *Cronobacter* PubMLST. Most strains had the *fliE-R* gene cluster except *C. turicensis* ST5 strain 564, which was negative for *fliI* and *fliG* while still

showing motility. Conversely, the absences of *fliG* and *fliN in C. universalis* type species 581 resulted in no motility observed for this strain. However, all of these genes were present in *C. sakazakii* ST8 strain 513 which was non-motile. Further screening was performed for presence/absence of *flgN-L* gene cluster (ESA_02264–77) and they were positive in all *C. sakazakii* isolates. *FlgK* and *flgE* were absent in *C. condimenti* strain1330 while *flgJ* and *flgI* were not found in *C. turicensis* ST5 strain 564 which are motile, while the non-motile *C. universalis* strain 581 was only missing *flgJ.* Furthermore, all of bacterial isolates were positive for *fliT* and *fliz* genes that are responsible for motility in *Salmonella enterica* and described by Frye *et al,* (2006). However, Choi *et al,* (2014) indicate that Plasmid-Encoded *mcp* regulates the motility in *C. sakazakii* ATCC 29544, (*C. sakazakii* ST8 strain 1) which is the type species in NTU collection. This gene was present only in this strain. All strains have twitching motility protein *pilT* gene (Yan *et al,* 2011), which may explain the ability of some strains to move throughout the medium although the absence of some *fli* or *flg* genes despite twitching motility being associated with movement on solid not liquid medium.

Presence of 70% and above of total number of nucleotides of each gene was considered as positive in this study.

3.3.6 Capsule production.

3.3.6.2 Capsule production on Xylose lysine Deoxycholate agar (XLD) medium.

Capsule production on XLD medium can be divided into four categories dependent on the amount of capsule produced. In general, most strains were able to produce high levels of capsule material and were categorized in highly capsulated group. Three strains, *C. sakazakii strain* 709, *C. universalis* strain 581 and *C. malonaticus* strain 546 produced moderate amount of capsule. *C. malonaticus strain* 681 and *C. sakazakii* strain 513 were low capsule producers. Strains *C. sakazakii* strains 696, 701, 716, and 1, and *C. malonaticus* 1569, *E. coli* K1 strain 939, *E. coli* K12 strain 1230 and *S. Enteritidis* strain 358 were non-capsulated according to the morphological appearance on this medium (Table3-3 and Figure 3-11).

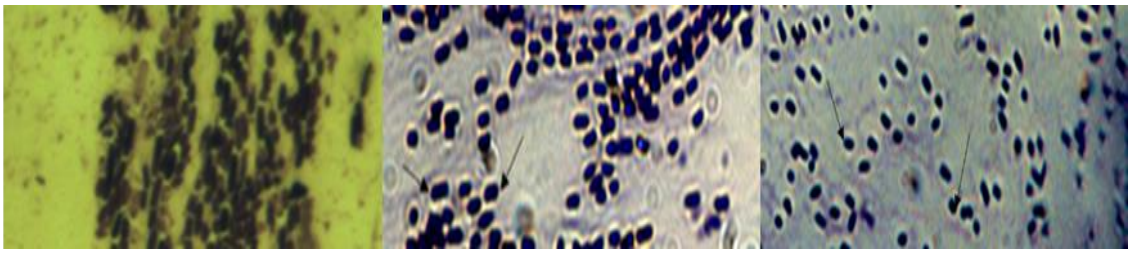
3.3.6.3 Capsule production on Violet Reed Bile Glucose Agar (VRBGA) medium.

This medium containing glucose sugar as one of the ingredients, and is used in capsule production assay to compare with results from XLD medium which contains xylose, lactose and sucrose as carbon sources. Results were varied from non-capsulated to highly capsulated as it indicated in table 3.3. On this medium, only *Cronobacter sakazakii* type species isolate 1 was unable to produce any capsule along with non *S. enteritidis* 358 and *E. coli* 1230. *C. sakazakii* strains 696, 701, 716, and *C. malonaticus* strain 1569 they showed low levels of capsules formation on this medium and were categorised as a low capsule producers based on the colony morphology on this medium.

3.3.6.4 Capsule production on milk agar medium.

Bacterial isolates were investigated for the production of mucoid material (capsules) on agar medium supplemented with two different milk formula (Cow & Gate and SMA 1 First infant milk) separately. Obtained results from these two-different media were almost identical. However, the *C. malonaticus* strain 1558 produced low levels of capsule on Cow and Gate (C&G), while it was non-capsulated on SMA medium. *E. coli* 1230 K12 a negative control in tissue culture studies continued to be non-capsulated on all type of examined medium, while a *E. coli* K1 strain 939 produced moderate levels of capsules on both milk medium. Moreover, a positive control in tissue culture experiment *S. enteritidis* strain 358 showed very low capsule production on milk agar based medium while it was non-capsulated on XLD and VRBG medium.

Moreover, capsule production was confirmed by Anthony's capsule staining method in order to visualise the capsule material (Figure 3.11), which can be seen as a halo around the bacterial cells**.** However, although it was possible to observe the halo around the capsulated strains, the size of the halo surrounding the bacterial cell does not reflect the amount of mucoid capsule material produced by bacteria when grown on soled medium.



C. Sakazakii 716 non capsulated | *C. Sakazakii* 767highly capsulated | C. Malonaticus 1558 moderate capsulated

Figure 3-11 Anthony's staining method showing the capsule as a halo around the bacterial cells.

Table 3-3 Summary of capsule production on different agar medium of *Cronobacter* isolates and non *Cronobacter* isolates *E. coli* K1 939, *E. coli* 1230 and *Salmonella* Enteritidis 358.

Media	Non-capsulated	Low capsulated	Medium capsulated	High capsulated
XLD	**696**[a], **701**[a], 716, 1, **1569**[c], *E. coli K1* 939, *E. coli* 1230 and *S.* Enteritidis 358	681 513	**709**[b] 581 and 1546	693, 700, **703**[a], **767**[c], 1210, **1211**[d], 1330, 1561, 978, **1249**, 563, 1557, 1558, **564**[c] and 1556
VRBG	1, *E. coli* 1230 and *S.* Enteritidis 358	**696**[a], **701**[a], 716, 1557 ,681, 1558, **1569**[c] and *E. coli K1* 939	700, 581,**1249**[c], 513 and 1546	693, **703**[a], **709**[b], **767**[c] , 1210, **1211**[d], 1330, 1561, 978, 563, **564**[c] and 1556
C&G	*E. coli* 1230, 358	**696**[a], 1558 1556	716, 513 ,**1249**[c], **1569**[c] and *E. coli Ki* 939	693, 700, **701**[a], **703**[a], **709**[b], **767**[c] , 1, 581, 681, 1210 , **1211**[d], 1330, 1561, 978, 563, 1557, 1546 and **564**[c]
SMA	1558, *E. coli* 1230, S. Enteritidis 358	1556, **696**[a]	716, 513, **1249**[c], **1569**[c] *and E. coli K1 strain 939*	693, 700, **701**[a], **703**, **709**[b], **767**[c], 1, 581, 681, 1210 , **1211**[d], 1330, 1561, 978, 563, 1557, 1546 and **564**[c]

Isolates in bold are clinically significant. **a:** isolates obtained from neonates with NEC strains, **b:** Trachea isolate from neonate septicaemia **c:** isolates obtained from neonates with Meningitis, and **d:** fatal isolates

3.3.6.5 Genes involved in capsule production.

Genes involved in the capsule synthesis in *Cronobacter* were described by Ogrodzki and Forsythe, (2015). *Cronobacter* isolates were searched for the presence/absence of the genes related to capsule production. According to a recent paper about *Cronobacter* capsule profiling, *kps* K-antigens consist of three regions where most, if not all strains share genes in regions 1 (kpsEDCS) and 3 (*kpsTM*), and differ in region 2. While K-antigen type 1 (K1) have three genes *HP1*, *epsJ* and *cps1,* K-antigen type two (K2) group have *HP2, epsH* and *cps2.* Strains were varied accordingly and divided into two groups, k1 and K2. Colanic acid genes were varied and categorized into two groups CA1 (21 genes) and CA2 (20 genes), which can be discriminated by the absence of *galE* gene from CA2 group (Ogrodzki and Forsythe, 2015)

The outcome of genomic screening was highly varied, especially between *kpsEDCS* genes group. *kpsED* was 100% present in all investigated isolates while *kpsC* was positive in most strains except *C. malonaticus* type species (681) and *C. universalis* type species (581). Interestingly, *kpsS* was missing in all *C. sakazakii* except ST1 strain 658, ST8 strains, ST14 strain 716 and ST23 strain 563. Region 3, *kspTM* were present in all strains.

Furthermore, *galE* was chosen as indicator for CA1 or CA2 groups and screened in all strains. Strains that were found to be positive for *galE* were considered as CA2, while those do not have this gene considered CA1 (Ogrodzki and Forsythe, 2015). Most of strains were from CA2 group (69%). Among *C. sakazakii* strains, ST3, ST4, ST12, ST13, ST31 and ST8 type strain NCTC 11467 (NTU ID 1) are CA2, while isolates from ST1, ST8 strain 513, ST14 strain 716 and ST 31 strain 1557 was CA1. Only *C. malonaticus* strain 1569 isolate was CA2 (Table 3-4). Additionally, *C. turicensis* strains 564 and 1211, *C. dublinensis* strain 1556, C*. condiment* strain 1330 and *C. universalis strain 581* were belonged to CA2 group.

However, when the genomic research results were compared with experimental results there was no obvious link between presence/absence of these genes and the amount of capsule obtained. Only *C. sakazakii* 696 missing *kpsS* (ESA_03352) and all of the region 2 in from *kps* gene, produced a very little amount of mucoid material. In general, K-antigen type seems not to effect capsule biosynthesis, as both K-antigen types were able to produce a detectable amount of mucoid material.

However, in this study 66% of *C. sakazakii* strains have capsule profile K2:CA2:Cell+ described by Ogrodzki and Forsythe (2015), and among these isolates 70% were considered clinically significant and associated with serious neonatal infections such as meningitis, septicaemia or NEC. Among *C. malonaticus*, only strain 1569 has this profile and it was associated with neonatal meningitis in this species.

Together K-antigen, CA and cellulose genes, strains have capsule profile K2:CA2:Cell+ showed moderate to high capsule production, especially in milk based medium, suggesting the role of medium nutrient in capsule production including source of carbon and nitrogen that highly found in infant formula milk.

Table 3-4. Presence/absence of capsule, colanic acid and cellulose genes

NTU ID	Species	ST	Capsule Biosynthesis gene (kps) antigenes							Cellulose genes									Colanic acid group
			Region 1				K-antigen type	Region3											
			kpsE	*kpsD*	*kpsC*	*kpsS1*		*kpsT*	*kpsM*	*bscA*	*bcsB*	*bcsC*	*bcsE*	*bcsF*	*bcsZ*	*bcsQ*	*bcsG*	*yhjR*	
658	*C. sakazakii*	1	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA1
978		3	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
984		3	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
701		4	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
707		4	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
709		4	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
767		4	+	+	+	-	K1	+	+	+	+	+	+	+	+	+	+	+	CA1
1		8	+	+	+	+	K1	+	-	+	+	+	+	+	+	+	+	+	CA2
513		8	+	+	+	+	K2	+	+	+	+	-	+	+	+	+	+	+	CA2
696		12	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
703		12	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
693		13	+	+	+	-	K2	+	+	-	-	-	-	-	-	-	-	-	CA2
700		13	+	+	+	-	K1	+	+	-	-	-	-	+	-	-	-	-	CA1
716		14	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA2
1557		23	+	+	+	+	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
1249		31	+	+	+	-	K2	+	+	+	+	+	+	+	+	+	+	+	CA1
563		41	+	+	+	-	K1	+	+	+	+	+	+	+	+	+	+	+	CA1
1558	*C. malonaticus*	7	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA1
681		7	+	+	-	+	K1	+	+	+	+	+	+	+	+	+	-	+	CA1
1546		84	+	+	+	+	K2	+	+	+	+	+	+	+	+	+	+	+	CA2
1569		307	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA2
564	*C. turicensis*	5	+	+	+	+	K2	+	+	+	-	-	-	+	+	+	+	+	CA2
1211		19	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA2
1556	*C. dublinensis*	+	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA1
1210		106	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA2
1330	*C. condiment*	98	+	+	+	+	K1	+	+	-	-	-	-	-	+	+	+	+	CA2
581	*C. universalis*	54	+	+	-	+	K1	+	+	+	+	-	-	+	+	-	+	+	CA2
1561	*C. muytjensii*	81	+	+	+	+	K1	+	+	+	+	+	+	+	+	+	+	+	CA2

KpsE (ESA_03349): Capsule polysaccharide export inner-membrane protein, ***KpsD***: Capsular polysaccharide export system periplasmic protein, ***kpsC:*** Capsule polysaccharide export protein, ***KpsS***: Capsule polysaccharide export protein, ***CapC***: Capsule biosynthesis protein, ***KpsT:*** olysialic acid transport ATP-binding protein, ***KpsM:*** colanic acid capsular biosynthesis activation protein A, ***bcsA***: cellulose synthase catalytic subunit, ***bcsB:*** cellulose synthase, ***BcsC:*** *cellulose* synthase subunit ***bcsE***: cellulose biosynthesis protein, ***bcsF***; Cellulose biosynthesis protein, ***bcsZ:*** endo-1,4-D-glucanase***, bcsQ***: Cellulose biosynthesis protein (membrane protein), ***bcsG*****:** hypothetical protein (cellulose biosynthesis proteins), ***yhjR*****:** hypothetical protein and **ST:** sequence type.

Table 3-5 capsule profiling of selected strains according based on, Colanic acid and Cellulose genes and K-antigen serotype

NTU ID	Species	ST	K-antigen type	Colanic acid type	Cellulose bcs genes	O-type	Capsule profile	Cellulose Genes
658	*C. sakazakii*	1	K1	CA1	cel^{+}	Csak O1'	K1:CA1:Cell+	*bcsC*
978		3	K2	CA2	cel^{+}	Csak O2	K2:CA2:Cell+	
984		3	K2	CA2	cel^{+}	Csak O2	K2:CA2:Cell+	
701		4	K2	CA2	cel^{+}	Csak O2'	K2:CA2:Cell+	
709		4	K2	CA2	cel^{+}	Csak O2	K2:CA2:Cell+	
767		4	K2	CA2	cel^{+}	Csak O2'	K2:CA2:Cell+	
1		8	K1	CA1	cel^{+}	Csak O1	K1:CA1:Cell+	
513		8	K1	CA2	cel^{+}	Csak O4	K1:CA2:Cell+	
696		12	K2	CA2	cel^{+}	Csak O4	K2:CA2:Cell+	
703		12	K2	CA2	cel^{+}	Csak O4	K2:CA2:Cell+	
693		13	K2	CA2	cel^{+}	Csak O2'	K2:CA2:Cell+	
700		13	K2	CA2	cel^{-}	Csak O2'	K2:CA2:Cell-	
716		14	K1	CA1	cel^{-}	Csak O1'	K2:CA2:Cell-	
1557		23	K1	CA2	cel^{+}	Csak O2	K1:CA2:Cell+	
1249		31	K2	CA2	cel^{+}	Csak O2	K2:CA2:Cell+	
563		41	K2	CA1	cel^{+}	Csak O3	K2:CA1:Cell+	
1558	*C. malonaticus*	7	K1	CA1	cel^{+}	Cmal O2	K1:CA1:Cell+	*bcsG*
681		7	K1	CA1	cel^{+}	Cmal O2	K1:CA1:Cell+	
1546		84	K1	CA1	cel^{+}	Cmal O2	K1:CA1:Cell+	
1569		307	K2	CA2	cel^{+}	Cmal O1	K2:CA2:Cell+	
564	*C. turicensis*	5	K1	CA2	cel^{+}	Ctur O4	K1:CA2:Cell+	*bcsBCE*
1211		19	K2	CA2	$cel^{+/-}$	Ctur O1	K2:CA2:Cell+/-	
1556	*C. dublinensis*	88	K1	CA2	cel^{+}	Cdub O2	K1:CA2:Cell+	*bcsA-C and E*
1210		106	K1	CA1	cel^{+}	Cdub O1b	K1:CA1:Cell+	
1330	*C. condiment*	98	K1	CA2	cel^{+}	Cuni O1	K1:CA2:Cell+/-	*bcsA-F*
581	*C. universalis*	54	K1	CA2	$cel^{+/-}$	Cuni O1	K1:CA2:Cell+/-	*bcsCE*
1561	*C. muytjensii*	81	K1	CA2	cel^{+}	Cmuyt O2	K1:CA2:Cell+	*bcsA-G*

CA: colonic acid; **Cel+** : cellulose genes present; **Cel**- cellulose genes absent, **bcsA:** cellulose synthase catalytic subunit, ***bcsB****:* cellulose synthase, ***BcsC***: cellulose synthase subunit ***bcsE****:* cellulose biosynthesis protein, ***bcsF***; cellulose biosynthesis protein, ***bcsZ***: endo-1,4-D-glucanase, ***bcsQ*****:** cellulose biosynthesis protein (membrane protein), ***bcsG***: hypothetical protein (cellulose biosynthesis proteins), and **ST:** sequence type

3.3.7 Blood haemolysis assay

This assay was performed to investigate whether the bacterial strains can produce haemolytic enzymes that can either rupture the red blood cells (beta haemolysis) or oxidise the iron in the haemoglobin (alpha haemolysis). This can be noted from the zone around the bacterial colonies. Results indicated that most of the investigated strains showed clear zones around the colonies similar that obtained from the beta haemolysis positive control *Streptococcus pyogenes* (NCTC 9994). *C. sakazakii* NTU number 693 morphology on horse blood agar was more greenish, which is considered alpha (α) haemolysis similar to that, obtained with alpha haemolysin control *Staphylococcus aureus*. No haemolytic activity was observed by *C. sakazakii* NTU number 1, *C. muytjensii* strain 1561, *C. malonaticus* strain 1546 and *C. dublinensis*. Moreover, the non *Cronobacter* isolate *E. coli* K1 strain number 939 which is a possible meningitic strain showed non-haemolytic ability on horse blood agar medium which is (γ) haemolysis. In contrast, to this result, none of investigated strains were able to haemolyse the sheep blood and all were considered as gamma (γ) haemolytic strains with respect to sheep blood. Examples shown in figure 3-12.

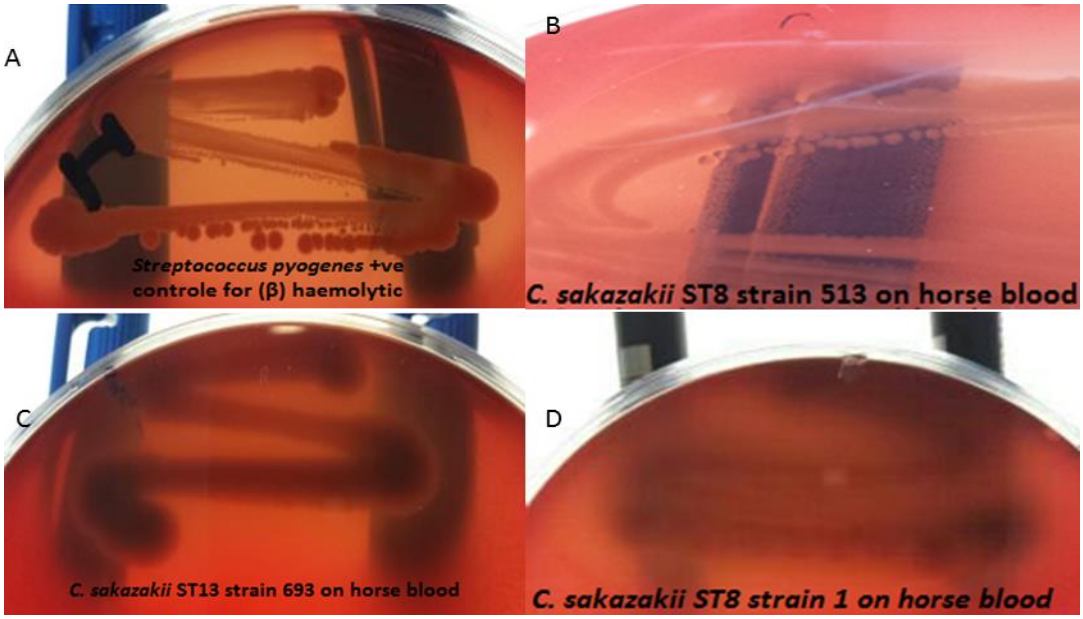


Figure 3-12 Bacterial haemolytic activity on horse blood after 24hr incubation at 37°C. Bacteria from 16-24h TSA plates were streaked on pre-prepared horse blood agar plates as described in section 2.8.4.10. **A**: the positive control for beta haemolysis, **B**: *C. sakazakii* ST8 strain 513 beta haemolytic, **C**: *C. sakazakii* ST13 strain 693 alpha haemolytic and **D**: non-haemolytic *C. sakazakii* ST8 strain 1.

3.3.7.2 Haemolysin Associated Genes

Genes responsible for haemolysis (*hly*) in *Cronobacter* were previously described (Hana Sonbol 2015, unpublished data), which are: haemolysin activator protein precursor (ESA_00102), predicted membrane protein haemolysin III homologue (ESA_00432), haemolysins and related proteins containing CBS domains (ESA_00643), haemolysin expression modulating protein (ESA_02810), putative haemolysin (ESA_02937) and a haemolysin precursor (ESA_03540). However, A BLAST search showed that all strains harbour these genes, although some strains showed non-haemolytic activity on both blood types. This might be because the expression of these genes in those strains is regulated by other genes, or mutants occurred during the evolutionary progress of non-haemolytic strains.

3.3.8 Biofilm formation.

Biofilm formation by pathogenic microorganisms on the equipment and processing environments surfaces, is the key source of contamination in powder infant formula and other food products. The genus *Cronobacter* have the ability to develop biofilms on different surfaces like stainless steel, silicon, polycarbonate, latex, and many equipment using at processing places (Iversen *et al,* 2004b; Lehner *et al,* 2005). This experiment was performed using Cow & Gate and SMA 1 First infant milk for only strains selected for tissue culture experiments based on their pathology site, ST and other virulence factors stated previously in this chapter.

3.3.8.2 Biofilm formation on plastic surface using 24-well plates.

Bacterial strains from 18h culture were diluted to OD_{600} 0.05 in Cow and Gate and SMA First Infant Milk, and then incubated in 24-well plates for 24h at 37^{o}C and subsequently stained as described in section 2.8.4.9.1. About 96% of isolates (27/28) produced more biofilm in C&G milk than that observed from SMA milk.

There is a clear variation between strains. Meningitic *C. malonaticus* Strain 1569 was the highest biofilm producer in both C&G and SMA milk. *C. sakazakii* strains 716 and 701, and *C. malonaticus* strain 681 showed biofilm range from OD_{540}1.8 to 2.02. Conversely, *C. malonaticus* strain 1546 was almost unable to develop any biofilm on both milk

medium, while strains *C. turicensis* 564 *C. sakazakii* strain 563, 693 and 716, and *C. malonaticus* strain 1558 displayed very low amount of biofilm on SMA milk medium with OD_{540} less than 0.03 (Figure 3-13 and 3-14). This phenomenon was also observed with *E coli* isolates K12 strain 1230 (non-invasive strain) and K1 strain 939 both showed OD_{540} of biofilm production in C&G milk higher than that produced obtained from SMA milk. (Figure 3.14).

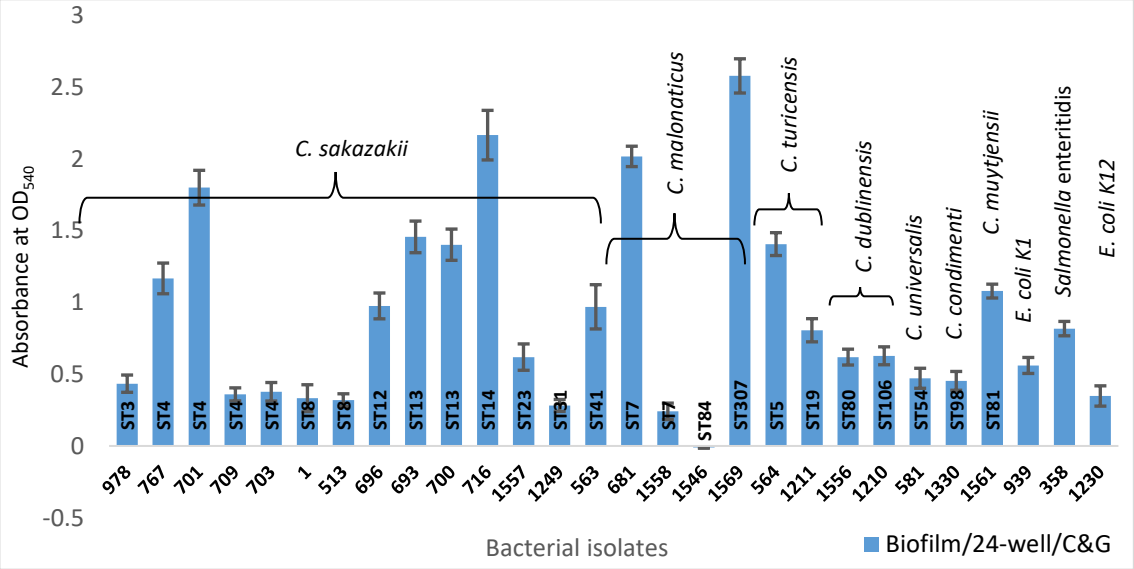


Figure 3-13 Biofilm formation on 24-well plates after 24h incubation in C&G First Infant Milk. Numbers inside or above the columns indicating to strain sequence type. Data shown as a mean of three experiments with error bars showing standard deviation. The OD of blank where no bacteria added subtracted from obtained results

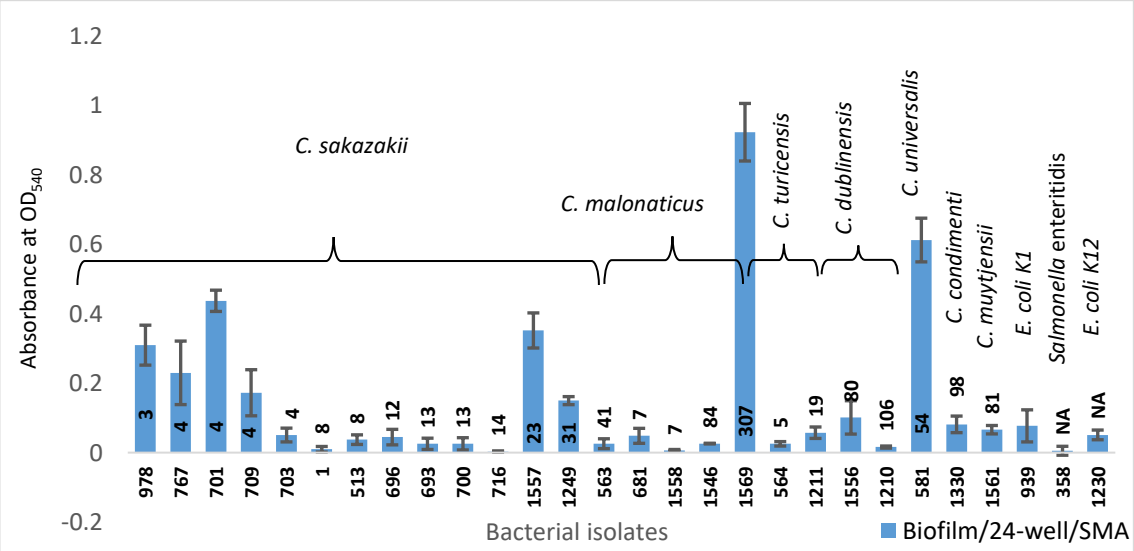


Figure 3-14. Biofilm formation on 24-well plates after 24h incubation in SMA First Infant Milk. Numbers inside or above the columns indicating to strain sequence type. Data shown are the mean of three experiments with error bars showing standard deviation. The OD of blank where no bacteria where added was subtracted from obtained results.

However, strains showed statically significant (P<0.0001) in the biofilm produced in SMA and C&G milk using Graph pad prism/paired t test, and the pattern of amount produced does not match on both medium.

3.3.8.3 Biofilm formation on neonatal enteral feeding tube (EFT)

The biofilm assay was repeated using EFT, and bacterial isolates showed greater biofilm production on EFT with C&G milk (16/27) than was that noted with SMA (Figure3-15). Among *Cronobacter* isolates, *C. sakazakii* ST4 strain 767 and *C. malonaticus* ST 307 strain 1569 were the highest biofilm producers on EFT when C&G milk was used, with optical density of up to OD_{540} 1.45. These two stains showed less biofilm production when SMA milk was used. *C. sakazakii* ST4 strains 701 and 709 and ST13 strain 693 were shown to be high biofilm producers compared with other isolates from same species. *C. sakazakii* strains 978 and 513 ST 8 and *C. malonaticus* 1546 were the lowest biofilm producers on EFT in C&G milk with recorded OD_{540} about 0.2 and less. Interestingly, the highest biofilm produced on EFT at C&G milk was revealed by the non *Cronobacter* isolates. *E. coli* K1 939 and *Salmonella* enteritidis showed OD_{540} 1.7 and 1.5 respectively (Figure 3.15). Overall, strains with serious pathology displayed biofilm from C&G milk more than that showed when SMA milk was used. Some strain such as *C. malonaticus* strain 1558 and *C. sakazakii* strain 709 produced higher biofilm in SMA higher than C&G milk. The OD of biofilm production of the possible meningitic strain *C. sakazakii* 767 and *E. coli* K1 strain 939 from SMA milk was only 0.67 and 0.49 compared with 1.46 and 1.72 for C&G milk respectively (Figure 3.16).

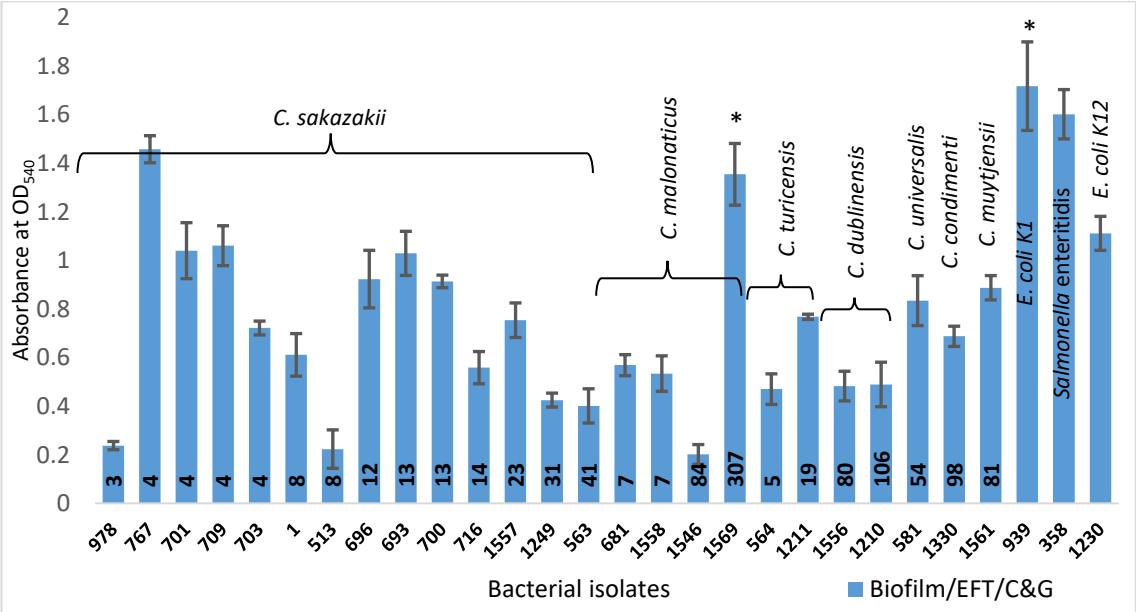


Figure 3-15 Biofilm formation on enteral feeding tubes after 24h incubation in C&G First Infant Milk. Numbers inside the columns indicating to strain sequence type. Data shown are the mean of three experiments with error bars showing standard deviation. The OD of blank where no bacteria were added was subtracted from obtained results. The asterisks above the bars indicate to suggested meningitic strains

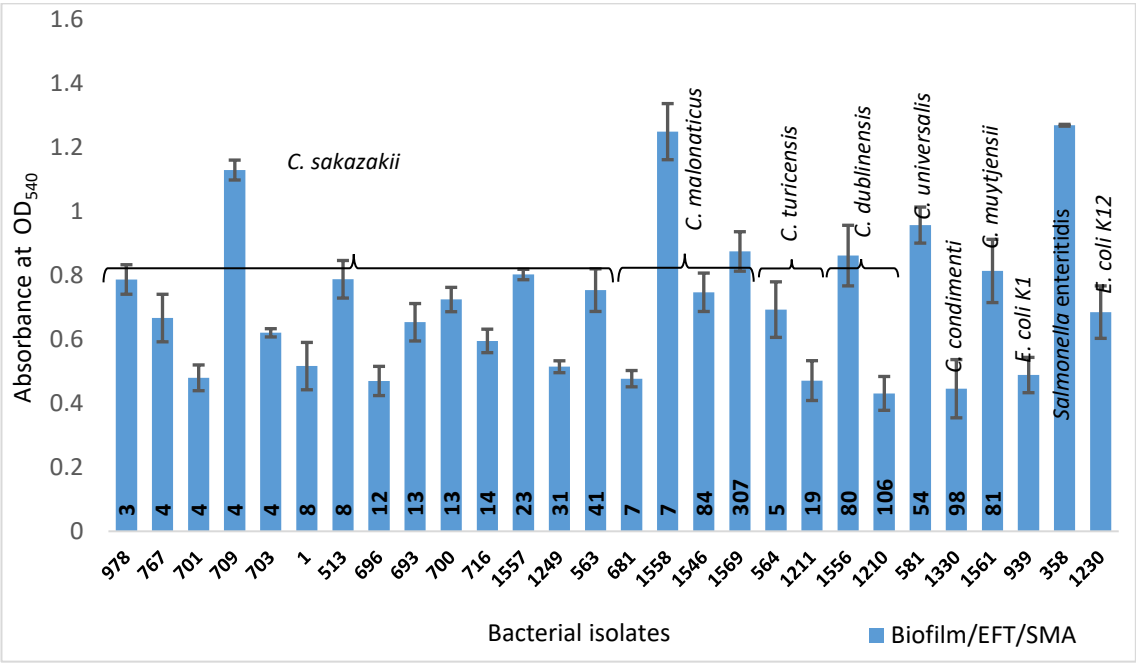


Figure 3-16 Biofilm formation on enteral feeding tubes after 24h incubation in SMA First Infant Milk. Numbers inside the columns indicating to strain sequence type. Data shown as a mean of three experiments with error bars showing standard deviation. The OD of blank where no bacteria added was subtracted from obtained results. The asterisks above the bars indicate to suggested meningitic strains

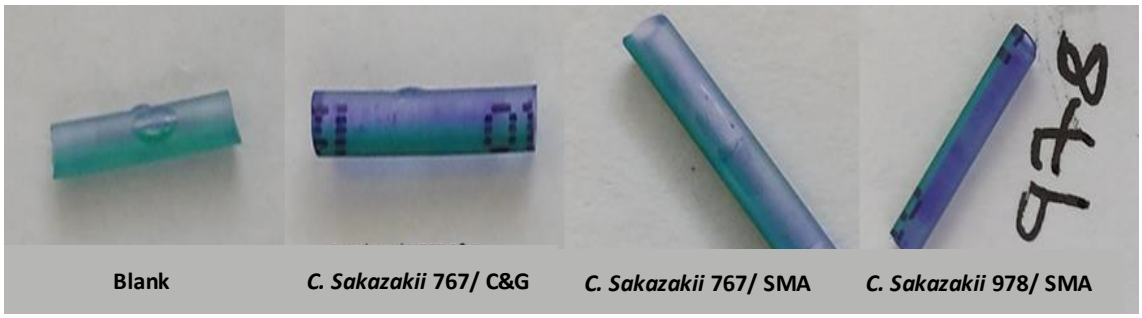


Figure 3-17. Bacterial biofilm on enteral feeding tubes incubated in ready to use First Infant Milk. 16-24h broth culture diluted in milk medium to OD_{600} of 0.05, and four pieces of EFT (1cm each) were incubated for 24hr at 37°C. EFT, were then washed with PBS several times, air dried and stained with 1% crystal violet for 30 minutes. Dye was then extracted with ethanol and OD taken at 540nm. Blank: no bacteria added

Importantly, the results obtained from biofilm experiment indicated that almos all of investigated strains were able to produce biofilm on EFT in considerable amounts. This suggests that these strains if allowed to settle in such places for an appropriate period, might be able to produce biofilms that provide protection against unsuitable environmental conditions.

3.3.8.4 Genes responsible for biofilm formation

Using *Cronobacter* BLAST search, isolates were searched for gene encoding the hypothetical proteins ESA_00281, ESA_00282 described by Hartmann *et al,* (2010) and *flhE, fliD,* and *flgJ*, possibly genes involved in biofilm formation (Hartmann *et al,* 2010 & Ye *et al,* 2015).

Data obtained from BLAST indicated that *fliD, flag,* ESA_00281 and ESA_00282 were present in all strains, while *flhE* was absent in *C. universalis* type species strain 581. Cellulose production is involved in biofilm formation, and expressed by *bcsA-G and yhjR* genes (table 3-4) which were recently described by Ogrodzki and Forsythe (2015), and random mutation on these genes were found to affect the biofilm and cellulose production (Hartmann *et al,* 2010; Hu *et al,* 2015). A BLAST search was performed for this gene cluster in twenty-six strains. This cluster was completely absent in ST13 (strains 693 and 700) with with exception of *bcsF* that was found in strain 700, yet these strains produced biofilm. *C. sakazakii* ST8 strain 513 do not have *bcsC, while C. turicensis* ST5 strain 564 was missing three genes which are *bcsB, bcsC, bcsE. C. condimenti* ST98 strain 1330 was negative for genes *bcsA-F* (5/9) and *C. universalis* negative for *bcsBC* and *bcsF*.

Generally, no correlation was observed between the lab experimental results and the absence or presence of investigated genes, which suggests that different genes might involve in this process. Finally, our results indicated that the amount of obtained biofilm was most likely depend on the type of material and mill formula used in this assay.

Table 3-6 summary of strains that missing dsome genes.

Reference			Hartmann *et al.,* 2010		Ye *et al.,* 2015)						
Isolate	species	ST	ESA_00281	ESA_00282	*bcsC*	*bscA*	*bcsG*	*fliD*	*flhE*	*flgJ*	*yhjR*
513	C. sakazakii	8	+	+	-	+	+	+	+	+	+
564	C. turicensis	5	+	+	-	+	+	+	+	-	-
581	C. universalis	54	+	+	-	+	+	+	-	+	+
1330	C. condimenti	98	+	+	-	+	+	+	+	+	+
693	C. sakazakii	13	+	+	+	-	-	+	+	+	-
700	C. sakazakii	13	+	+	+	-	-	+	+	+	-
713	C. sakazakii	13	+	+	-	-	-	+	+	+	-
714	C. sakazakii	13	+	+	-	-	-	+	+	+	-
715	C. sakazakii	13	+	+	-	-	-	+	+	+	-

3.3.9 Outer membrane protein profiling.

Outer membrane proteins (OMPs) play important roles in bacterial pathogenicity through improving bacterial adaptations in the surrounding environment, thus production of OMPs in Gram-negative bacteria is mostly regulated by environmental conditions (Lin *et al,* 2002). Summary of the results included in this chapter is stated in table 3-7.

Therefore, bacterial strains that were associated with significant clinical symptoms were chosen to investigate the variation in their OMPs production at 37^{o}C in broth medium. Results indicated that there is a clear variation in the number and molecular wight of the OMPs (Figure 3-18). Most strains produced OMPs ranging from 10Kda to about 75KDa. There was a variation between strains from same species and even same ST group. *C. sakazakii* ST3 strain 978 has an extra OMP at about 70KDa, which is missing from strain 984 from same ST. Moreover, ST4 isolates grouped in different clusters, strains 701 and 709 located in separate sub-cluster, whereas strain 767 was an individual in outlier cluster with OMP size more than 75Kda that also found in *C. sakazakii* ST8

isolate 1 and 513. In contrast, *C. sakazakii* 658 and *C. malonaticus* 681 clustered together with 11 OMPs similar.

E. coli K1 strain 939 has only 7 OMPs ranging from 10KDa to about 65KDa shopwing an obvious differences from the size of *Cronobacter* OMPs

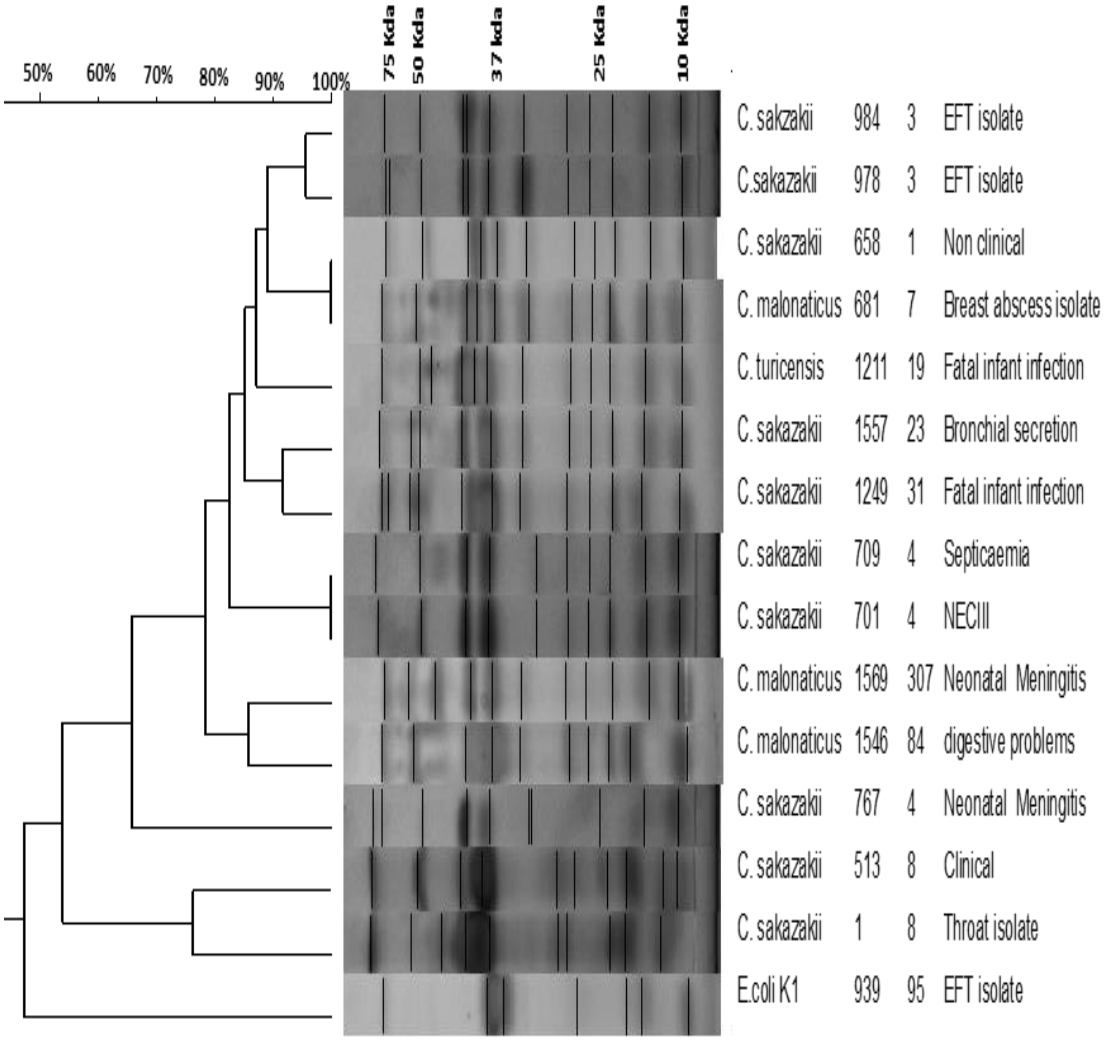


Figure 3-18. Outer membrane proteins (OMPs) profiling for selected clinically important strains. The analysis was performed using BioNumerics software, version 6.5. Dice's coefficient and the unweighted pair group method with arithmetic mean (UPGMA) were used for cluster analysis. ST, sequence type. Dendrogram clusters were defined at a 50 % similarity level, while sub-clusters were defined at a 70% similarity level. Cluster 1 had two sub-clusters, one for *C. sakazakii* strain 1 and another cluster for strain 513 from same species and ST8. Cluster 2 had 4 sub-clusters. The first includes *C. sakazakii* strains 984, 978, 658, and *C. malonaticus* strain 691, the second includes *C. sakazakii* 1557 and 1247, the third comprised two *C. sakazakii* ST4 strains 701 and 709, while the last cluster include *C. malonaticus* strains 1569 and 1558 plus one outlier strain *C. sakazakii* 767.

Table 3-7. Summary of physiological traits results of selected *Cronobacter* isolates, that will be further investigated in host pathogen interaction experiments

NTU No	Species	Source	ST	Country	Capsule				Motility mm	activity on HB	Biofilm fromation				Highest media for capsule production
											Biofilm on plate		Biofilm on EFT		
					SMA	C&G	VRBG	XLD			SMA	G&G	G&G	SMA	
978	*C. sakazakii*	EFT isolate.	**3**	UK	H	H	H	H	**56**	B	L	L	M	L	All high
767		Meningitis	**4**	French	H	H	H	H	**69.6**	B	M	M	M	H	All high
701		NECIII	**4**	French	H	H	L	N	**71.6**	B	L	H	M	H	SMA & C&G high
709		SEPTICEMIA	**4**	French	H	H	H	M	**68.3**	B	L	L	H	H	SMA, C&G and VRBG high
703		NECII	**4**	French	H	H	H	H	**67**	B	VL	L	M	M	All High
1		Throat isolate	**8**	USA	H	H	N	N	**60**	γ	VL	VL	M	M	SMA, C&G type high
513		Clinical	**8**	Czech Republic	M	M	M	L	**0**	B	VL	VL	M	L	SMA, C&G and VRBG moderate
696		NECII	**12**	French	M	M	L	N	**62.3**	B	VL	M	M	H	SMA, C&G and VRBG moderate
693		Asymptomatic	**13**	French	H	H	H	H	**79.3**	α	VL	L	M	H	All high
700		Digestive Problem	**13**	French	H	H	M	H	**80**	B	VL	H	M	M	SMA, C&G and XLD moderate
716		Non clinical	**14**	French	M	M	L	N	**66**	B	VL	H	M	M	SMA, C&G moderate
1557		Bronchial secretion	**23**	Unknown	H	H	L	H	**71**	B	L	M	M	M	SMA, C&G and XLD high
1249		Fatal infant	**31**	UK	M	M	M	H	**83.5**	B	VL	VL	M	M	XLD high
563		Foot wound isolate	**41**	USA	H	H	H	H	**73**	B	L	M	M	L	All high
681	*C. malonaticus*	Breast abscess isolate.	**7**	USA	H	H	L	L	**76.5**	B	VL	H	M	M	SMA, C&G high
1558		Sputum isolate	**7**	Czech Republic	L	L	L	H	**41.5**	B	VL	L	H	M	XLD high
1546		digestive problems	**84**	Czech Republic	H	H	M	M	**36**	B	VL	VL	M	L	SMA, C&G high
1569		Blood isolate	**307**	USA	M	M	L	N	**79**	B	M	H	M	H	SMA, C&G moderate
564	*C. turicensis*	Blood isolate	**5**	USA	H	H	H	H	**78**	B	VL	M	M	M	All high
1211		Fatal infant infection	**19**	Switzerland	H	H	H	H	**79.5**	B	VL	M	M	M	All high
1556	*C. dublinensis*	Abscess - base of spine	**80**	USA	L	L	H	H	**74.5**	B	VL	M	M	M	VRBG and XLD high
1210		Non clinical	**106**	Ireland	H	H	H	H	**72**	B	VL	M	M	M	All high
581	*C. universalis*	Non clinical	**54**	UK	H	H	M	M	**65**	B	M	M	M	M	SMA, C&G high
1330	*C. condimenti*	Non clinical	**98**	Slovakia	H	H	H	H	**73.5**	B	VL	M	M	M	All high
1561	*C. muytjensii*	Non clinical	**81**	USA	H	H	H	H	**76.5**	B	VL	M	M	M	All high
939	*E. coli* K1	EFT isolate	**95**	UK	M	M	L	N	**25**	γ	VL	M	L	H	C&G moderate
	E.coli K12	negative control	NA	Unknown	N	N	N	N	**4**	α	VL	L	H	H	All non-capsulated
	Salmonella enterit	Positive control	NA	Unknown	N	N	N	N	**52**	β	VL	M	M	H	All non-capsulated

ST: sequence type**; NECI, II, III:** necrotizing enterocolitis stage 1,2 and 3**; NA**: not applicable, **L**: low; **M**: moderate, **H**: high and **VL**: very low, **N**: non-capsulated, **SMA**: Synthetic Milk Adaptation-first Infant milk ready to use formula, **G&G:** Caw and Gate-first Infant milk ready to use formula, **VRBG:** Violet Red Bile Glucose**, XLD**: Xylose lysine deoxycholate agar, **EFT:** enteral feeding tube, **NEC**: necrotizing enterocolitis type II or III.

3.4 Discussion.

Contamination with pathogenic microorganisms is a serious concern for almost all types of environments. Published data clearly indicate that there is an association of *C. sakazakii* outbreaks with reconstituted PIF (Himelright *et al*, 2002; Jarvis, 2005; Joseph and Forsythe, 2014), with predominant clonal complex four (CC4) (Hariri *et al,* 2013; Sonbol *et al,* 2013; Fei *et al,* 2015). This group of microorganisms was found to persist for long periods in food industry environments like powdered infant formula (Iversen *et al,* 2008; Jung *et al,* 2013). Phenotypic and physiological characters of pathogenic bacteria such as motility, capsule and biofilm formation are some of the most studied bacterial features nowadays as these characteristics have significant roles on bacterial pathogenicity (Houry *et al,* 2009; Chellappa *et al,* 2013 and Kalai Chelvam *et al,* 2014)

3.4.1 Bacterial growth rate

Pathogenic bacterial growth rate in particular environment is an important factor in the pathogenicity. The loss of nutrient concentration and accumulation of secondary metabolites, pushing bacteria to reveal more competition, and produce extracellular toxins to kill other bacterial cells, this correspondingly might have serious effects on the host tissues in case of infection. The multiplication of pathogen in the gut lumen and the differences between dead and new cells might be one of the important factors in bacterial survival and the disease development (Malin and Paoletti, 2001).

Although all of bacterial isolates diluted to same optical density (OD), there was clear variation in their growth curves. All strains showed an exponential increase in the first 9hrs. The differences of obtained OD between the investigated strains at this point (9hrs) were the highest. Some strains revealed nine folds duplication of the initial while some strains showed only five times fold. In regard of the *Cronobacter* seven type species result which stated in (Figure 3.1), *C. dublinensis* strain 1556 was the highest when OD increased from 0.05 to 0.45 while the density of *C. muytjensii* strain 1561 showed less than five folds' increase.

The maximum recorded OD for the seven-type species was between 24-28 hrs (OD= 0.34-0.35). After 28 hrs, strains started to reveal a gradual decrease in the turbidity, which might be due to the lysis of bacterial cells. The growth curve of the other group of *C. sakazakii* isolates (Figure 3.2) which were chosen according to their clinical history, displayed same profile of growth. The exponential rate trended to move up to stationery phase after 9 hours growth with a slight increase over the next 15h. *C. sakazakii* 1557 showed the highest OD at 9 and 24 hours while neonatal asymptomatic isolate 693 presented the highest growth level at 28 and 32 hrs.

The highest recorded OD for *Cronobacter* spp isolates was obtained by *C. dublinensis* 1556, which was 0.458 at 24h of growth (Figure 3.3), this strain was the highest growing strain between 3 to 32 hours. However, *E. coli* K1 strain 939 recorded the highest OD among all investigated strains in the first nine hours. This result allowed to confirm that all strains used from 14-24 broth culture in this project obtained from stationary phase.

Differences of growth rate among the strains propose that strains showed sharp exponential increase in their growth rate such as *E. coli* K1 939, *C. dublinensis* 1556, *C. sakazakii* 1557 might be able to reveal more competition in the subsistence environment and production of toxins and secondary metabolites, which can affect host cells and contribute disease.

As many protocols state an initial concentration of bacteria added to host cells, determining how this inoculum alters during the typical 3h duration of co-culturing experiment is important. To achieve the similar tissue culture environment conditions, bacterial growth rate was further investigated in Dulbecco's Modified Eagle Medium (DMEM), the common used in tissue culture medium at conditions similar to that at tissue culture experiments. Results are presented as the change in OD over 3h in antibiotic-free tissue culture medium and inoculum of OD_{600} 0.05 (Figure 3.4, 5 and 6). Our finding are broadly in agreement with Sezonov *et al,* (2007) suggesting that the manner and capability of continuing bacterial growth is medium and nutrients dependant.

The highest obtained OD among *Cronobacter* type species was displayed by *C. malonaticus* strain 681, which was the least growth on TSB, followed by strains 1211 *C. turicensis* and *C. muytjensis* 1561, which they were not among the highest growing strains in TSB. *C. sakazakii* ST4, ST8 and ST12, which associated with the most serious clinical signs in neonates, revealed high growth productivity and duplicated by 7-9 folds of the inoculum. However, this may explain the association of these particular ST groups with diseases like necrotising enterocolitis (NEC) in neonates and meningitis, as the conditions used in this experiment mimic the host conditions.

The high population of the bacteria might lead to increases of the inflammatory response in neonates due to the immaturity of their immune system sometimes (Niño, Sodhi, and Hackam, 2016). However, these differences in growth rates could subsequently lead to variation on bacterial metabolites such as exotoxins production that affect the host cell defences mechanisms and enhance pathogen to cause disease.

In general, although some strains which had high growth rate were in addition among the high capsulated, motile and biofilm producers, the correlation between growth rate results and other phenotypical character of other strains is not clear. For example, *C. sakazakii* strain 513 showed high growth rate, but was non-motile and moderate to low biofilm producer. Moreover, there was no clear relation between the growth rate and other investigated virulence factors as we expected, for example, strain 513, showed highest increase in the OD and it was one of the lowest adhesive strains and IL-8 production stimulators, and moreover, this was observed with *C. turicensis* strains 564 and *C. malonaticus* strain 1546. These results will be further discussed in the chapters of host pathogen interactions.

Our results confirmed that bacterial cell numbers increase at different rates, which should be taken into account when looking at interaction with tissue culture cells. Bacterial strains may start at same OD, but 3h later some strains increased 3-fold while others increased 6 fold and more.

3.4.2 Phenotypic characterization of bacterial isolates.

Morphological study of the selected strains on different agar medium showed that strains differs even among same sequence type, results are shown on table (Table 3-6). Bacterial binding to Congo red dye is indicator to the ability of pathogenic bacteria to bind to the host hemin and protoporphyrin IX that are required for the oxygen-carrying capacity of haemoglobin and are structurally similar to the aromatic dye Congo red (Daskaleros and Payne 1987; Palma *et al,* 1994, Avendano-Herrera *et al,* 2005 and Ruiz *et al,* 2016). This character is assumed to be an evidence of expression of curli fimbriae in *Salmonella* Isolates (Finn *et al,* 2013) and *E. coli* (Lloyd *et al,* 2012 and Reichhardt *et al,* 2015).

Congo red assay performed at 37^{o}C, 28^{o}C that approximately simulating the body and skin temperatures respectively (Hillion *et al,* 2013). The most presented colony phenotype obtained was brown, and smooth (BAS) which was 50% and 73% at 37^{o}C and 25^{o}C respectively. This phenotype however, according to the description of Yan *et al,* (2015) not able to bind to Congo red, and from other hand, these strains not able to express curli fimbriae and bind to human hemin and protoporphyrin. RAS, the weak binding to Congo red was more observed at 37^{o}C (19%) than 28^{o}C (2%) which might indicate to the role of temperature in bacterial virulence. However, RDAR or BDAR morphotypes which are positive for the Congo red dye binding assay (Yan *et al,* 2015) were comparatively less than BAS and RAS. RDAR composed 21% and 17% at 37^{o}C and 28^{o}C respectively, while BDAR was only 10% and 45% at 37^{o}C and 28^{o}C respectively.

Most of the strains that showing either RDAR or BDAR were clinically significant. Among *C. sakazakii* ST4 strains, only strain 701 which is a neonatal fatal NECIII isolate showed strong binding to Congo red dye at both temperatures and was categorised as RDAR. This phenotype was also noted by *C. malonaticus*, the neonate fatal meningitic isolate. *C. sakazakii* ST3 isolates showed strong binding to Congo red dye at 37^{o}C which was more obvious with strain 978 that showed a strong cytotoxicity to epithelial cells that not shared with all of other ST3 isolates. However, there was no association of this phenotype with specific species or sequence type (see table 3-2)

Some of *Cronobacter* species harbour curli fimbriae genes (Joseph *et al,* 2012), and binding to Congo red is an indicator of the expression of curli fimbriae in different bacterial species, which is very important structure for binding to host cells, and other virulence factors such as biofilm formation (Finn *et al,* 2013; Hung *et al,* 2014; Kalai *et al,* 2014). However, there is no published data reported that *C. sakazakii* have these genes or can express this structure (Joseph *et al,* 2012 and Forsythe *et al,* 2014). However, Congo red is also binding to cellulose polymers expressed by some pathogenic bacteria such as *E. coli*, which is possibly miss-identify as a Congo red positive (Hung *et al,* 2014), which is on the other hand meaning that phenotypical characterisation is important for initial identification, but not completly accurate and further steps are vitally needed to correctly identify certain pathogens.

3.4.3 Acid stress assay.

Bacterial acid resistance is a clinically important phenotype despite the lack of any clear correlation with the pathogenicity (Foster, 2004). Several studies indicated that different *Cronobacter* spp. isolates were able to establish infection in neonates and adults and attach, invade and translocate through different human cell lines in *in vitro* based studies (Iversen *et al,* 2008; Joseph *et al,* 2012a and Forsythe 2014, Jackson *et al,* 2014 and Almajed and Forsythe, 2016). This genus is very sensitive to the possible excessive pH (2.2 ± 0.02) of neonatal gastric acid (Hurrell *et al,* 2009), and success of escaping this extreme condition and establishing infection can be considered as another virulence factor of this genus.

Previous data indicated that this genus is able to growth and even display an exponential increase at pH 3.5 and above (Dancer *et al,* 2009), while some of other pathogens can persist at pH 2.5 or even lower such as *Shigella* and *E. coli* (Gorden and Small. 1993; Lin *et al,* 1995). The average of gastro intestinal pH is 1-5 (de Jesus *et al,* 2005; Hurrell *et al,* 2009 and Alvarez-Ordóñez *et al,* 2014). In neonates, the minimum recommended time between each two feeds is 2 hrs (8-12 times/24 hours) (Boskabadi *et al,* 2010), thus, fasting stomach could have a period of pH as low as 1-2 (Smith, 2003) which is not real obstacle for those known to cope with low pH. Although, this genus is very sensitive to

the possible excessive pH 2.2 ± 0.02 used in recent study, that mimic the fasting neonatal gastric acid (Hurrell *et al,* 2009), it was able to escape this extreme conditions and establish infection, which can be considered as another virulence factor of this genus.

As none of the investigated strains were able to survive at pH of 2.2, suggesting that *Cronobacter* have different mechanisms to avoid exposure to such conditions, either by biofilm formation (Welin-Neilands and Svensater. 2007) on the gastric tubes, or by mechanisms of rapid invasion and translocation immediately after ingestion during the conditions of the gastric acid pH is 3.5 or higher. Genetically, *Cronobacter* isolates are lacking acid resistance related genes (*gadA, gadB, gadC, hdeA, hdeB* and *hdeB*) found in *E. coli* and described by (Masuda and Church, 2002), and the recent study reported the ability of *E. coli* K1 strain 939 and *E. coli* K12 to grow at pH of 2.2 suggesting the important role of these genes. Finally, as indicated by Hurrell *et al,* (2009) that neonatal gastric pH can decline up to 2.5 after consumption of breast milk, the pH largely inhibits microbial growth, it can be advised that breastfeeding can help in protection from *Cronobacter* infections. However, investigation of the ability of the pathogenic bacteria to form biofilms in low acid environment is eventually required to determine the role of milk type in biofilm formation and neonatal protection.

3.4.4 Bacterial motility.

The motility of bacterial isolates was also investigated. Flagella are the organelles that are responsible for swimming/ swarming motility, and contribute to the adherence and invasion of host tissues. Motility is further linked to bacterial cell translocation, and plays an important role in the host immune response (Hartmann *et al,* 2010, Amalaradjou and Venkitanarayanan. 2011; Haiko and Wikström. 2013). Experimental results indicated that most of tested strains (95%) were able to show a detectable motility on the semisolid medium, and there was a considerable variation in the motility levels between the investigated strains. The highest level of motility was showed by clinically significant strains such as *C. sakazakii* ST31 strain 1249 which is a fatal infant infection isolate, and ST4 strain 730 which is a faecal isolate from a neonate who was suffering from

necrotizing enterocolitis (NEC I), *C. turicensis* strains 1211 and 564 which are fatal infant infection and blood isolates respectively.

Moreover, based on motility, *C. malonaticus* strains divided were into two groups, strains isolated from patient with severe infection were highly motile which are strain 681 (breast abscess isolate) and 1569 (blood isolate -Fatal meningitis), while the other two strains1546 and 1558 that are bed swab and faecal isolate showed moderate motility. These results may indicate to the role of bacterial motility and disease occurrence and development. Interestingly, all of *C. sakazakii* ST13 isolates were highly motile in this assay as shown in Figure 3-8 and 3-9 despite no link to disease, while there is no relationship between the motility and other group of ST. For example: *C. sakazakii* ST8 strain 1 showed a motility zone of 60 mm motility while strain 513 from the same group of ST was non-motile although both are clinical isolates. Likewise, *C. malonaticus* strain 681 showed about 75 mm motility while 1558 revealed only 41 mm, although both are from ST7 group. However, different genes could be involved in bacterial virulence including motility and biofilm. For example. ompR receptor could influence bacterial flagella motility and biofilm formation via regulating of *ompF* and *ompC* genes in *E. coli* (Raczkowska *et al,* 2011; Ye *et al,* 2015), which is possibly that these mechanisms are also applied in *Cronobacter*.

Genes associated with flagellar proteins in *Cronobacter* which were previously described by Kucerova *et al,* (2010) and Joseph *et al,* (2012a) were searched in all of the investigated strains using BLAST in *Cronobacter* MLST database. No obvious correlation was observed between motility results and presence or absence of *fli* (*fliR-E*) and *flg* (*flgL-N*)) genes, and for example *C sakazakii* ST8 strain 513 was non-motile although it is positive for these two clusters. All strains were positive for pili protein *pilT* gene, it is possible that bacteria can use pili in twitching motility, and it is possible that this gene not expressed in non-motile strains. Interestingly, the recent study of Hoeflinger and Miller, (2017) indicated that some *Cronobacter* strains although they harbour flagellar genes *flhA*, and *fliG* were non-motile and do not express flagella, suggesting involvement of different genes and cell components such as pili and fimbriae in bacterial motility (Conrad *et al,* 2011).

In general, there was no clear correlation between the *in vitro* and *in silico* result, suggesting a possible role of different genes in bacterial virulence, which supports the finding of Choi *et al,* (2014) about *mcp* gene that was found to regulate different bacterial virulence factors including motility, and mutant strains revealed an enhanced expression in the flagellar genes.

Bacterial motility is involved in different virulence traits such as biofilm formation, and may contribute in bacterial adhesion and invasion to mammalian cells, and may stimulate host cells to produce hyper inflammatory response that could leading to autoimmune disease, and subsequently increasing the chance of the bacteria penetrating into bloodstream and cause further diseases. Generally, strains showing high motility also displayed other virulence characteristics such as high attachment and IL-8 production, which will be discussed further in chapter 5**.** Some strains with high motility revealed high biofilm production on Cow and Gate milk in both 24-well plates and enteral feeding tubes such as *C. sakazakii* stains 701 which is a possiblly responsible for neonatal death due to sever necrotizing enterocolitis, and 716 the IF isolate from the same outbreak. Moreover, motility was observed to contributeto biofilm formation by *C. malonaticus* strains 1569 and 681, which are also clinically significant (see table 2-1).

3.4.5 Biofilm production.

Biofilm production is one of important bacterial pathogenic characters that gives bacteria the ability to escape and avoid the environmental stresses. Bayles (2007) pointed out that programmed death of bacterial cells is very important for biofilm formation as the new bacteria can lyse the dead cells and release genomic DNA, which has an essential role in the adhesion between cells and biofilm stability. Bacteria that can produce biofilm may be able to survive in acidic and bile salts environments similar to that in human stomach, and resist elevated temperature and antibiotics (Ito *et al,* 2009 & Healy *et al,* 2010; Abdel-Aziz and Aeron 2014).

Cronobacter isolates were found to develop biofilm on neonatal enteral feeding tubes (Kim *et al,* 2006), that might be inoculate following routine feeds into the neonate's stomach as clumps of cells protected by biofilm structures, which may be a key of

Cronobacter survival in neonate's stomach in contrast of planktonic cells that showed high sensitivity to low pH.

The results of biofilm formation of the selected strains indicated that 96% of strains were able to produce biofilm on polystyrene surfaces (24-well plates) using C&G milk first infant (ready to use), while only 75% when SMA milk was used first infant (ready to use). There is no correlation between the sequence type and biofilm production, and as an example, the possible meningitic ST4 *C. sakazakii* 767 showed the highest biofilm production among *Cronobacter* isolates in enteral feeding tube using C&G milk compared with about half displayed by ST4 *C. sakazakii* strain 703.

Generally, strains associated with disease were able to produce greater biofilm than others in C&G milk, and interestingly, the possible meningitic *E. coli* K1 strain 939 showed the highest level of biofilm production on EFT using C&G milk among all of tested strains. The importance of biofilm is that bacterial cells in biofilms structure are more resistance to antimicrobial agents (Percival *et al,* 2011), and the anionic polysaccharide barrier could deactivate antimicrobial mediator s (Shigeta *et al,* 1997). Thus, biofilm cells may require a minimum inhibition concentration of antibiotic up to 1000 times higher than planktonic cells (Chen and Wen, 2011). Moreover, infections associated with bacterial biofilm are not resolved by immune system (Woods *et al,* 2010). This may explain how bacteria can survive and translocate from the site of infection to bloodstream and then to central nervous system and cause meningitis in neonates.

C. malonaticus strain 1569 showed the highest level of biofilm formation in 24-well plates with C&G milk among all *Cronobacter* isolates. However, in C&G milk, *C. sakazakii* strain 716 and *C. malonaticus* strain 681 were able to form biofilm in 24-well plates greater than that observed in enteral feeding tube, which is in contrast with SMA when they produced biofilm considerably higher on the enteral feeding tubes than that obtained from 24-well plates. *E. coli* 939 and 1230 strains revealed much lower biofilm on 24-well plate using SMA milk compared with that obtained from 24-well plate and C&G milk, indicating to the role of milk ingredients on biofilm production.

Generally, these results support the finding by Kim *et al,* (2006) of the ability of *Cronobacter* spp to form biofilm in enteral feeding tube and other surfaces, which is one of the possible virulence traits responsible for bacterial resistance to antimicrobial agents such as antibiotics and bile salts as well as heat tolerance and osmotic stress (Healy *et al,* 2010). However, most of investigated strains were able to form biofilm in both milk types with varied amounts, and overall biofilm production using C&G milk which is about 100% whey based was higher than that displayed in SMA which is casein based. These results indicated that the milk ingredients play an important role in biofilm production and subsequently bacterial survival in extreme environmental conditions such as acid stress in neonatal stomach and as a result the disease occurrence.

All strains were searched for the potential genes involved in biofilm formation, and described in previous publications in different bacterial species. No correlation was observed between the assay results and genetic research, as the biofilm obtained was most likely depend on site of binding and formula used in this assay. However, the mismatching of experimental results and genome BLAST research might refer to the gene expression, as some genes might non-expressed or regulated by different genes

3.4.6 Capsule production.

Ogrodzki and Forsythe (2015) reported that capsule type can be diverse from one strain to another, and capsular typing scheme can be applied to detect a potential virulence trait associated with neonatal infections. Their study reported that *C. sakazakii* ST3*,* CC4 and ST12 isolates have capsular profile K2:CA2:Cell+. According in their study, *Cronobacter* isolates with this type of capsule are most associated with serious infections in neonates such as meningitis and NEC. This capsule type may be found in different STs and species in *Cronobacter* genus*.*

Interestingly, *C. sakazakii* ST31 strain 1249 and *C. malonaticus* ST307 strain 1569 both strains have capsular profile K2:CA2:Cell+, and are associated with fatal meningitis in neonates, which is broadly in agreement with this hypothesis despite no link with serotype or species. This result strongly suggests the possible role of capsule type in bacterial pathogenesis and disease development, which is possible that this capsule in

this genus offer an extra protection to the bacteria, and it also might enable the bacteria to evade the host immune mechanism, or even persist and replicate inside macrophages. This will be discussed further in chapter four in relation to host pathogen interaction.

Capsule production was investigated on different media, and the results indicated that most of tested strains were able to produce capsules with variable levels. As can be seen from table 3-3, most strains associated with pathologies were high capsulated, especially in milk based media such as 701, 1557 and 1569. In addition, some pathogenic strains such as 696, 701, 1, 1569, *E. coli* K1 939 were non-capsulated strains on XLD medium, but showed moderate to high capsule production on both milk media.

E. coli 1230 and *S.* Enteritidis 358 were completely non-capsulated in this study. This result supports the previous finding by Caubilla-Barron *et al,* (2007) who examined the French outbreak isolates and find that most of strains were able to form capsules on milk agar medium at considerable levels. However, *C. sakazakii* S13 strain 716 and all pulsed group four were described as a non-capsulated in their study, and showed moderate capsules on milk agars in present study. These results indicated that most of strains were more capsulate when infant formula used in the agar medium. Moreover, about 82% of the strains from neonates with serious pathology were highly capsulated on milk based medium, compared with only 50% and 40% when XLD and VRBGA medium respectively. This suggests the ability of bacterial isolates to utilize carbon and other capsule production requirement from milk easier than other type of substrates.

Genes responsible for capsule biosynthesis and exopolysaccharide colanic acid and cellulose production in *Cronobacter* were previously described (Ogrodzki and Forsythe 2015). These genes were searched in the tested strains in order to find any association with experimental results. However, no obvious link was observed between the presence/absence of these genes and phenotypes for the obtained capsule.

Generally, there was no link observed between K-antigen (K1 or K2) individually and capsule biosynthesis, while strains with capsule profile K2:CA2:Cell+ showed moderate to high capsule production, especially in milk based media, suggesting the role of the medium nutrient in capsule production including source of carbon and nitrogen that found in infant formula milk.

3.4.7 Haemolysis.

Haemolysing the blood cells by some pathogenic bacteria is an important virulence factor and plays an important role in pathogenicity of the onset of severe illnesses. (Wong *et al,* 2012). Certain infectious pathogenic bacteria can produce cytotoxic agents that destroy red blood cells. Infection with *Clostridium perfringens* that causes septicaemia subsequent to intra-abdominal infections and septic abortions is an example of blood haemolytic diseases due to release of alpha toxin, a phospholipase that lyses red blood cells membrane (Dhaliwal *et al,* 2004.

Haemolytic activity of the twenty-six strains on horse and sheep blood was investigated, and the finding indicates that most of *Cronobacter* strains were beta (β) haemolytic on horse blood except *C. sakazakii* strain 693 which was alpha (α) haemolytic according to the zone around the colonies, which was greenish. However, no haemolytic activity was observed by *C. sakazakii* strain 1, *C. muytjensii* strain 1561, *C. malonaticus* strain 1546 and *C. dublinensis* strain 1556. On the other hand, none of the investigated strains was able to lyse the sheep blood. Fakruddin *et al,* (2014) investigated 6 *Cronobacter* isolates and his finding showed that two of them were beta haemolytic. These results suggesting that the ability of some strains to lyse red blood cells could be one of the key virulence in survival of the bacteria in the bloodstream and causation of sepsis or meningitis.

Some of bacteria isolates that showed beta haemolysis were clinically significant, including those isolated from neonatal blood or causing meningitis such as *C. sakazakii* ST 41 strain 1249 and *C. malonaticus* ST307 strain 1569. The haemolysis activity expressed by these isolate to lyse the erythrocytes, could reflect the ability of strains to lyse different cell types and contribute to their invasion and distribution in host tissues.

Cronobacter genome includes six genes associated with haemolysis, (Joseph *et al,* 2012b). Twenty-six strains were investigated for the presence/absence of these genes and found to be all positive. However, some strains showed no haemolytic activity yet these genes were present, suggesting that these genes are not expressed in these strains or are regulated by other genes.

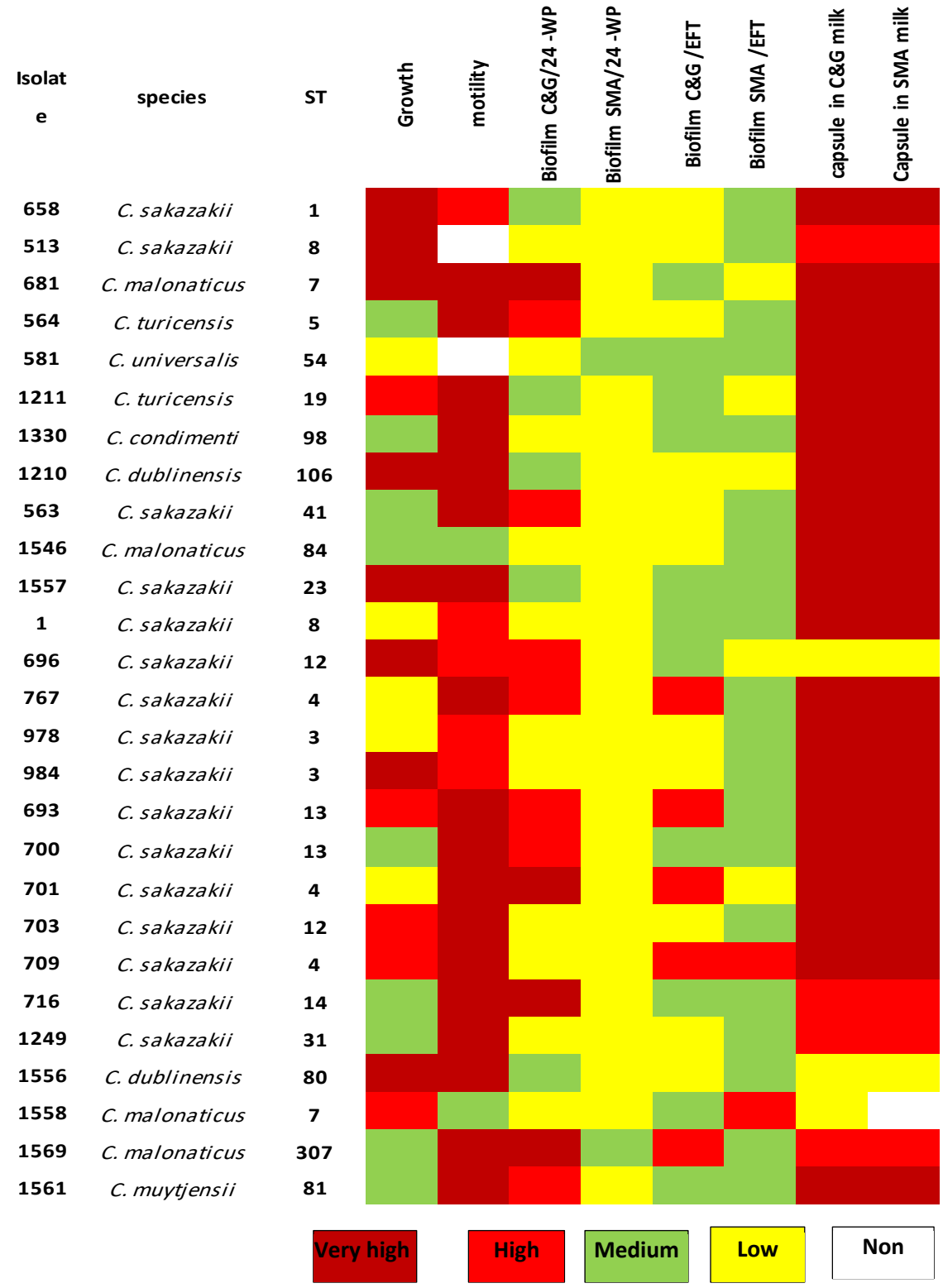


Figure 3-19. Summary of virulence traits investigated in chapter 3 and indicated as a heat map. **C&G:** Caw and Gate milk; **SMA**: Synthetic Milk Adaptation; **24-WP**: 24-well plate; **EFT**: Enteral feeding tube.

Chapter 4. H4 cell line: a new model of host pathogen interaction compared with Caco-2 cells

4.1 Introduction.

Despite the existence of multiple defence mechanisms displayed by hosts, pathogenic bacteria have developed a varied range of strategies to establish attachment and invade host tissues (Ribet and Cossart, 2015). Before birth, the neonatal gastrointestinal tract is considered sterile, and rapidly starts to colonize with different microorganisms during birth and from the surrouning environment (Patel *et al,* 2015). However, the early exposure to bacterial species could play a role in establishment of a stable colonic microbiota and development of immune system. (Patel *et al,* 2015). Colonisation of gastric intestinal mucosa by enteric bacteria begins instantly after birth, and within the first two weeks after delivery (Patel *et al,* 2015), and establishment of mature microbiota continues to grow over the period of 2 years of life (Patel *et al,* 2015; Moles *et al,* 2015).

However, preterm neonates are more at risk of being colonised by potentially pathogenic bacteria, mainly the multidrug-resistant bacteria due to the abnormal patterns of gastrointestinal microbiota development, and long hospitalisation compared with full term and healthy neonates (Chang *et al,* 2011; Moles *et al,* 2015). Pathogenic bacteria employ different mechanisms and produce variable molecules to facilitate their binding to the host cells and further utilize host responses to establish infection. These mechanisms differ from species to species and can include; attachment to the host cells surface, invasion, translocation to the underlying cells, and then diffusion into different organs (Wilson *et al,* 2002; Ribet and Cossart, 2015). However, the host epithelium is the key barrier to stop infection, and any loss of the integrity of this layer will facilitate invasion process and spreading into the tissues beneath (Kim *et al,* 2010; Ashida *et al,* 2012; Ribet and Cossart, 2015).

Cronobacter spp. Has been involved in many outbreaks in neonatal intensive care units (NICU), with reported serious pathologies including necrotizing enterocolitis (NEC), bacteraemia, and meningitis and high rate of mortality, and chronic disorders (Bowen

and Braden 2006; Mange *et al,* 2006; Caubilla-Barron *et al,* 2007; Hunter *et al,* 2008; Giri *et al,* 2011).

In France 1994, this organism was Involved in an outbreak where three neonates died due to necrotizing enterocolitis (NEC) two neonates and one neonate because of meningitis, and many others had severe pathologies included septicaemia and digestive disorders (Caubilla-Barron *et al,* 2007). Therefore, it was reclassified as an opportunistic pathogen (Healy *et al,* 2010). To create colonisation, invasion and consequent infection, *Cronobacter* spp. must adhere to the host cell membrane, and inhibition or blocking of this process might help in reducing infection (Quintero *et al,* 2011; Quintero-Villegas *et al,* 2014). Several studies reported the ability of *Cronobacter* ssp. to produce enterotoxins, attach and invade endothelial and epithelial cells *in vitro* such as HBMEC and Caco-2 cell lines (Pagotto *et al,* 2003, Mange *et al,* 2006). Townsend *et al,* (2007b) indicated that this bacterium could survive and replicate in human macrophage U937 cell line, and further strains from French outbreak were investigated and found to be able to attach and invade rBCEC4 and Caco-2 (Townsend *et al,* 2008). These results were confirmed when *C. sakazakii* isolates revealed adhesion and invasive ability into Caco-2, INT407, and HBMEC cell lines (Giri *et al,* 2011; Almajed and Forsythe, 2015).

The mechanisms beyond interaction of pathogens and establishment of disease is varied and not completely understood, and different pathogen-host components are involved. Type four pili (type IV pili) is one of pathogenic strategies and found to mediate different functions including adherence to eukaryotic cells and protein secretion (Aas *et al,* 2002; Reguera *et al,* 2005; Eva *et al,* 2010). However, fimbria are linked to adherence of *Cronobacter* spp. into Caco-2 and HEp-2 epithelial cell lines, and human brain microvascular endothelial (HBMEC) cell line (Mange *et al,* 2006). Kucerova *et al,* (2010) stated that GR8 cluster which contains seven genes encoding for pilus proteins was present in clinical *Cronobacter* ST4 isolate. Unfortunately, they do not indicate to the symptoms associated with this strain. Moreover, Whitfield, (2006) reported that colanic acid contributes to bacterial attachment to host cells. However, the genomic comparative study by Joseph *et al,* (2012) found that colanic acid encoding genes (ESA_01155-01175 and *wzABCKM*) were present in all sequenced *Cronobacter* strains.

Outer membrane proteins (OMPs) are another potential virulence factor that contribute attachment and invasion to host cells, and many Gram-negative bacteria possess different OMPs (Carlsohn *et al,* 2006; Galdiero *et al,* 2012). Both OmpA and OmpX were described as important factors for the basolateral invasion of *E. coli* and *Cronobacter* spp. to some mammalian cells such as spleen and liver cells and are thought to play important role in neonatal meningitis by helping pathogenic bacteria to translocate the blood-brain barrier (by Mittal *et al.*2009; Kim *et al,* 2010a). In addition, OmpA in *Cronobacter* spp. is found to aid in initiation of bacterial invasion to HBMECs through fibronectin-binding proteins (Mohan Nair *et al,* 2009). Moreover, several studies suggested the role of Type VI secretion system (T6SS) in influencing bacterial attachment, invasion, cytotoxicity and even growth inside macrophages and invasion of the human blood-brain barrier by *E. coli* K1, and five putative T6SS clusters were described in *Cronobacter*, (Kucerova *et al,* 2010; Franco *et al,* 2011; Joseph *et al,* 2012a; Zhou *et al,* 2012).

The recent study of study Du *et al,* (2016) indicated that specific genes in *Cronobacter* are involved in adhesion and invasion into human colon adenocarcinoma (HCT-8) cells line. These genes are: two flagellar biosynthesis genes (*flhA, AFK64_06715* and *fliR, ESA_02516*), a transcriptional regulator gene (*fnr, AFK64_08415*), exonuclease V subunit beta gene (*recB, AFK64_02315*), a PTS sugar transporter gene (*bglF, AFK64_20760*), as well as 3 genes encoding for hypothetical proteins which are: *hp*-1[a]: AFK64_01330, *hp*-2[a]: ESA_04202 and *hp*-3[a]: ESA_00132. Their study showed that the a mutant in these genes resulted in reduction in adhesion and invasion of *C. sakazakii* isolates up to 92, 89, 82, 89 and 97, % for the *recB, bglF, fnr, flhA and fliR* genes respectively, and arround 40% reduction obtained for *hp*-3a, ESA_00132 the hypothetical protein. (Du *et al,* 2016)

Bacterial translocation through intestinal epithelial tissues is a vital virulence factor, which allows pathogenic bacteria to distribute from the site of infection to different parts of the host body, and cause severe systemic disease (Hu *et al,* 2008; Wine *et al,* 2008; Pogačar *et al,* 2010). Some pathogenic bacteria interrupt the integrity of host epithelial cells to facilitate the translocation process by disrupting the tight junctions (TJs) and increasing cells permeability that help to translocation to underlying tissues (Wilson *et al,* 2002; Hsu *et al,* 2014). In addition, bacteria can translocate though the

accessible E-cadherin, a calcium-dependent cell-cell adhesion molecule (van Roy and Berx, 2008), which can facilitate rapid translocation, through transcytosis across enterocytes like that shown by *Listeria* spp. (Nikitas *et al,* 2011). *Shigella flexneri* can translocate by entering M cells that are known to take of antigens and microorganisms at the epithelial cells layer and help in initiation of immune response (Kucharzik *et al,* 2006), and reinvade epithelial cells basolateral, triggering the inflammatory response which could disrupts the epithelium integrity, thereby facilitating the translocation of additional bacteria (Perdomo, 1994). In addition, bacterial translocation can be also enhanced via activation of the TLR2/PI3K (phosphoinositide 3 kinase) pathway by bacterial infection, which can disrupt integrity of intestinal epithelial cells and facilitate bacterial penetration into deeper cells (Oppong *et al,* 2012)

However, since the main site of bacterial colonization is the intestinal tract, penetration of the epithelial layer and translocation to the bloodstream might lead to serious complications such as bacteraemia (Burns *et al,* 2001). Pagotto *et al,* (2003) and Mange *et al,* (2006) reported the ability of *Cronobacter* to produce enterotoxin, attach to, and invade Caco-2 and HBMEC cells in vitro. Moreover, a group of *C. sakazakii* isolates from the French outbreak in 1994 could attach and invade rBCEC4 and cell lines Caco-2, and persist and replicate in human macrophage U937 cell line (Townsend *et al,* 2007b; Townsend *et al,* 2008).

Furthermore, Giri *et al,* (2012) reported the adhesion and invasion of *C. sakazakii* to Caco-2, HBMEC, and INT407 cell lines. Townsend *et al,* (2007a) reported that the LPS from damaged bacterial cells in powdered infant formula (PIF), can increase epithelial permeability and facilitate pathogenic bacteria translocation including *C. sakazakii*, which was also reported by Giri *et al,* (2012) when *C. sakazakii* strains from different sites including meningitic isolate and blood isolate were able *in vitro* to translocate through the polarized monolayers of HBMEC and Caco-2 cell lines with frequency similar to that obtained by meningitis *E. coli* K1 strain which was used as a positive control in their study.

Bacterial motility and cytotoxicity are very important virulence traits that help bacteria to overcome host barriers (Alarcon *et al,* 2009). Increase of the cells death due to

secretion of cytotoxic compounds such as endotoxins including LPS, leading to loss of the cells integrity and increase the permeability, might help in the translocation through the host barrier (Hunter *et al,* 2008; Alarcon *et al,* 2009).

Cytotoxic activity of pathogenic bacteria to the host cell a very important factor in pathogenicity can be the result of variable factors and mechanisms (Alouf and Popoff 2005). Pore-forming toxins (PFTs) account for about 25% to 30% of bacterial cytotoxic proteins that can perforate the host cells membranes and other intracellular organelle membranes (Alouf, 2003; Iacovache *et al,* 2010). A study by Pagotto *et al*, (2003) demonstrated the 33% of clinical *Cronobacter* spp isolates were able to produce enterotoxins and only 12% of non-clinical isolates. The filtrates from these strains were found to be toxic to three cell lines which are: Chinese hamster ovary (CHO) cells, African green monkey kidney (Vero) cells, and adrenal gland; tumor/cortex-mouse. In addition to releasing of toxic compounds, cytotoxicity could happen because of haemolytic activity of haemolysins secreted by pathogenic bacteria.

Different genes were described to enhance cytotoxicity in pathogenic bacteria. Haemolytic activity was mostly linked to the presence of *hly* genes in *E. coli*, (May *et al,* 2000; Garcia *et al,* 2012; Mirsepasi-Lauridsen *et al,* 2016), and in *Cronobacter* (Cruz *et al,* 2011). In addition, different type secretion systems were involved in bacterial cytotoxicity to host cell. Type III secretion system (T3SS) was associated with a cute cytotoxicity caused by some pathogenic bacteria such as *Pseudomonas aeruginosa* (Wang *et al,* 2013) and *Aeromonas hydrophila* (Suarez *et al,* 2008). Pukatzki *et al,* (2006) mentioned that a mutation in some T6SS associated genes such as *vasH* and *vasK* genes in *A. hydrophila* showed less toxicity to human epithelial HeLa cells and murine macrophages. In *Cronobacter* spp T6SS was also involved in bacterium cytotoxicity (Hartmann *et al,* 2010; Franco *et al,* 2011; Kucerova *et al,* 2011; Joseph *et al,* 2012a). Yan *et al,* (2013) indicated that *C. sakazakii* genome possess the toxin-encoding protein *YkfI* which was reported in *E. coli* to regulate bacterial cell death processes (Brown and Shaw, 2003).

As stated, some pathogenic bacteria are capable of invading host eukaryotic cells, and use this process as a pathogenic mechanism. Rearrangement of host cell cytoskeleta0n

has been used by different enteropathogenic bacteria such as *Salmonella*, *Listeria*, *Shigella* and *Yersinia* for invasion process (Navarro-Garcia *et al,* 2013; de Souza Santos and Orth, 2015 and Mittal *et al,* 2016), and *Cronobacter* spp. (Mohan Nair and Venkitanarayanan 2007). Cytoskeletons consist of different proteins including microtubules that are made of large type of filamentous protein is tubulin, while microfilaments consist of smaller type protein known as actin (Mathur and Hülskamp, 2002).

Cytoskeletal actin is responsible for motility and shape in eukaryotic cells and many other essential cellular processes. Actin polymerization can be induced by some pathogenic bacteria such as *Listeria* and *Shigella* to propel the invaded bacteria from the cytoplasm into nearby cells (Cossart, 2000; Cossart and Sansonetti, 2004; Guiney and Lesnick, 2005; Ferreira *et al,* 2006 and Mittal *et al,* 2016).

Mohan Nair and Venkitanarayanan. (2007) investigated the role of human cytoskeleton inhibitors in the invasion of one *Cronobacter* spp. isolate into human intestinal (INT407) cell line. Their study performed by pre-incubation of INT407 cell line with microfilament (MF) inhibitor Cytochalasin D, and microtubule (MT) inhibitors vinblastine, Taxol Colchicine and Nocodazole. Their findings indicated that Cytochalasin D reduced the invasion by about 93%, while MT inhibitors reduced the invasion with range of 40%-17%. Moreover, a study by Mittal *et al,* (2014) showed that the inhibition of *Pseudomonas aeruginosa* invasion into human middle ear epithelial cells (HMEECs) by Cytochalasin D (MF) was dose dependent, and 10 µM concentration resulted in 98% reduction. Moreover, they found that 20 µM colchicine (MT inhibitor) reduced the invasion up to 92%, 85% by 20 µM Nocodazole and about 95% of the invasion was inhibited with 50µM Vinblastine. The effect of eukaryotic cytoskeleton inhibitors on the invasion of *Klebsiella pneumoniae* was further investigated by Hsu *et al,* (2014) who found that the invaded bacteria decreased to 8.5% with Cytochalasin D 0.1 µM and 30 µM Nocodazole.

The use of human and other eukaryotic cell lines for host-pathogen interaction research is globally extensively common. Cell lines simplify the complex *in vivo* processes, and may considerably shorten the period of research in contrast of the *in vivo* models (Law *et al,* 2013). Intensive amount of research was conducted in investigation of bacterial

pathogenicity mechanisms, and mostly focused on the host-pathogen interaction experiments. Different cell lines were established in which most of them were derived from cancer cells.

Caco-2 cell is one of the most popular *in vitro* used cell line in investigation of host-pathogen interaction in the gut that is described to exhibit a well-differentiated monolayer giving high similarity to the small-intestinal microvillus (Meunier *et al,* 1995; Buhrke *et al,* 2011), and exhibited spontaneous differentiation for long term culture (Sambuy *et al,* 2005;). However, Caco-2 cells are an abnormal cell line and are derived from an adult colon carcinoma (Buhrke *et al,* 2011) which may not match and mimic the pathogenesis mechanisms in neonates. Limited data are available about using non-malignant neonatal cell lines such as H4 cells in investigating the host pathogen interactions. Investigation of inflammatory responses indicated that neonatal cells are more responsive and producing more inflammatory cytokines than adult cells (Claud *et al,* 2003).

Non-transformed human cell lines will increase our understanding of epithelial cell function and mechanisms behind bacterial pathogenesis especially in neonates, particularly with disadvantages of genetic mutation of carcinoma cell lines that previously used in this field.

H4 cell line was established by Sanderson and Walker (1995) as new model of non-malignant human neonatal epithelia cells. Later, Sanderson *et al,* (1996) investigated some of the phenotypic characters of this cell line, and concluded that this cell line had the normal chromosome number (46+XY), and expressed villin and cytokeratins as proof of their epithelial cell source. Unlike other carcinoma cells such as Caco-2 or TE84, H4 cells do not form domes after reaching confluence, suggesting lack of sealing by tight junctions (Sanderson *et al,* 1996). H4 cell was then compared with adult Caco2 and HT29-cl19A in regard to their response to stimulation with IL-1β and TNF-α and IL-8 production, and showed IL-8 production significantly higher than both Caco2 and HT29-cl19A cell lines. (Claud *et al,* 2003).

Therefore, H4 cell was subjected to varied range of experimental investigation to determine whether this cell line can be better model in investigation of neonatal infectious diseases instead of transformed adult cell lines.

4.2 Material and methods

Methodology used in this chapter was described in chapter 2. However, a brief description is included as need.

4.3 Results

4.3.1 Optimization of Experimental Conditions

To the best of our knowledge, this is the first research investigating the human non-malignant neonatal intestinal H4 to for various host-pathogen co-culturing experiment, with different bacterial isolates from the seven species of genus *Cronobacter* and one *E. coli* k1 isolate strain 939 from Nottingham Trent University collection, thus the experimental conditions were first optimized.

4.3.1.1 Gentamicin sensitivity assay

All strains were investigated about their sensitivity to 125µg/ml gentamicin. No bacteria were recovered after 1-hour incubation in this concentration of antibiotic under tissue culture conditions similar to that applied for the host-pathogen interaction assay (data not shown)

4.3.1.2 Time required for confluence.

The required time for human non-malignant neonatal intestinal H4 cell line to reach confluence in 24-well plates was examined and compared with human colonic carcinoma epithelial (Caco-2) cell line. After cells were seeded according to recommended concentration 1 x 10^5 and 2 x 10^4 cells /ml respectively, the increase in the cell number was determined as well as the appearance of the cells under microscope was recorded.

Figure 4-1 showing the differences between the growth rates of both cell lines. I general H4 cell line was able to make about 100% confluent layer after about 18 hours, and about 70% within the first 12 hours. In contrast Caco-2 cell line lack to consist clear confluent monolayer on the 24-well after 24 hours and developed only about 50% confluence and 80% 90% after 48hrs.

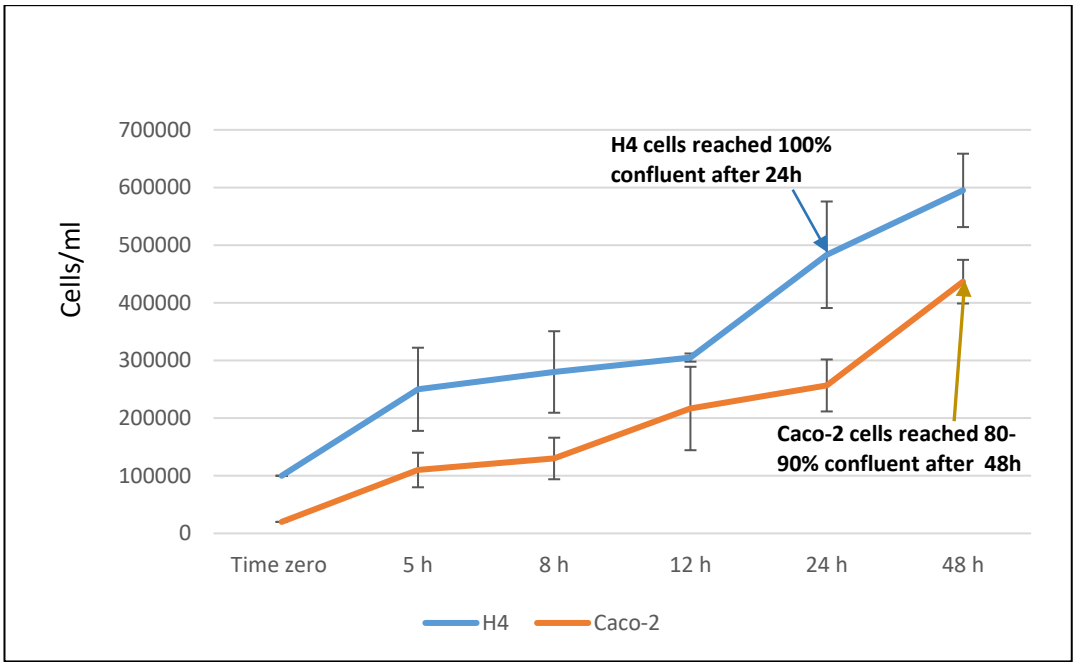


Figure 4-1 time require for 100% confluent by H4 and Caco-2 cell lines on 24-well plates. Human cells were seeded according to each protocol and cells were harvested at different time point to determine the growth curve for both cell lines to confluence. The displayed data are the mean ± standard deviation of the three replicates.

4.3.1.3 Role of passage number in bacterial invasion.

To determine whether the passage number of human cells has any effect on the bacterial virulence, selected strains were chosen for this assay. Falkow (1990) found that the invasion of some *Salmonella* isolates was about 10-fold lower on earlier passages of Madin-Darby Canine Kidney (MDCK) Cell. In a separate experiments, high H4 passage numbers (48-51) and low passages (17-19) were used in parallel to investigate the role of the passage number on bacterial invasion into human cells.

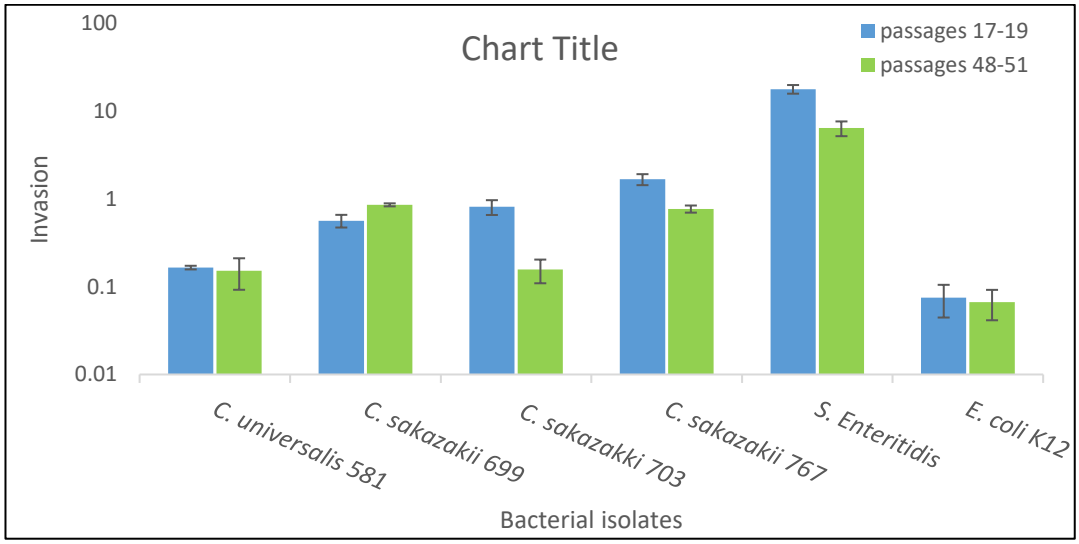


Figure 4-2. Invasion of *Cronobacter* isolates to low passages compared with high passages. H4 cells low passages (17-19) and high passages (48-51) were seeded in 24-well plates and incubated fro24h at 37°C and 5% CO_2. Then bacteria from 16-24h broth culture were diluted in tissue culture medium to OD_{600} of 0.05 and added to thes cells for 3h at 37°C and 5% CO_2 and number of invaded bacteria compared. The displayed data are the mean ± standard deviation of invasion efficiency as percent of the initial inoculum (MOI~1000) of three independent experiments in triplicate. No significant differences were found between passages using GraphPad prism/t test.

Results in figure 4.2 showing variable levels of invasion between strains to both high and low passages. A possible role of the passage level was observed with *C. sakazakii* strain 703 (NECII) and 767 the possible meningitic strain. However, in most cases differences were small. However, no significant differences were obtained using paired t test (P= 0.3008).

4.3.2 Attachment and invasion assay.

The attachment and invasion of the selected bacterial strains was investigated through the interaction of these strains with human non-malignant neonatal intestinal (H4) cell line passage (6-20). To the best of our knowledge, this is the first time for this cell line to be used in investigation of adhesive and invasive virulence of the seven *Cronobacter* type species or any other pathogenic bacterial species. The human colonic carcinoma epithelial cell line (Caco-2) was used in this part of the work to compare results obtained from H4 cells (passages 9-22). Results were determined after 3 hours of incubation of the mammalian cell lines with bacterial isolate after it was adjusted to an inoculum of OD 0.05 given multiplicity of infection (MOI) of approximately 1000 (Mooney *et al,* 2003). *Salmonella* Enteritidis (NTU strain 358) was used as positive control for all of the tissue culture experiment, whereas *E. coli* K12 (NTU strain 1230) was used as negative control.

4.3.2.1 Bacterial attachment to H4 and Caco-2 cells

The attachment of the bacterial strains to human non-malignant neonatal intestinal H4 cell line is shown in Figure 4.3. It can be seen that all strains were able to attach to this cell line in levels ranged from 1.6% of the inoculum as shown by *C. sakazakii* strain 693 (ST13) which is a faecal isolate from an asymptomatic neonate in French outbreak 1994 to 68.8% (P<0.001) which was showed by a *C. sakazakii* strain 563 (ST41) which is Foot wound isolate. *C. sakazakii* ST1 strain 658 which is non-clinical isolate was the second highest adhesive strain among *Cronobacter* isolates and showed about 59% from the inoculum. *C. sakazakii* ST12 strains 703 and 696 which were isolated from trachea and stools of two different neonates with necrotizing enterocolitis (NECII) and presented a significant results(P<0.001) compared with *E. coli* K12 and about 52% and 29% respectively, which were the highest among French isolates.

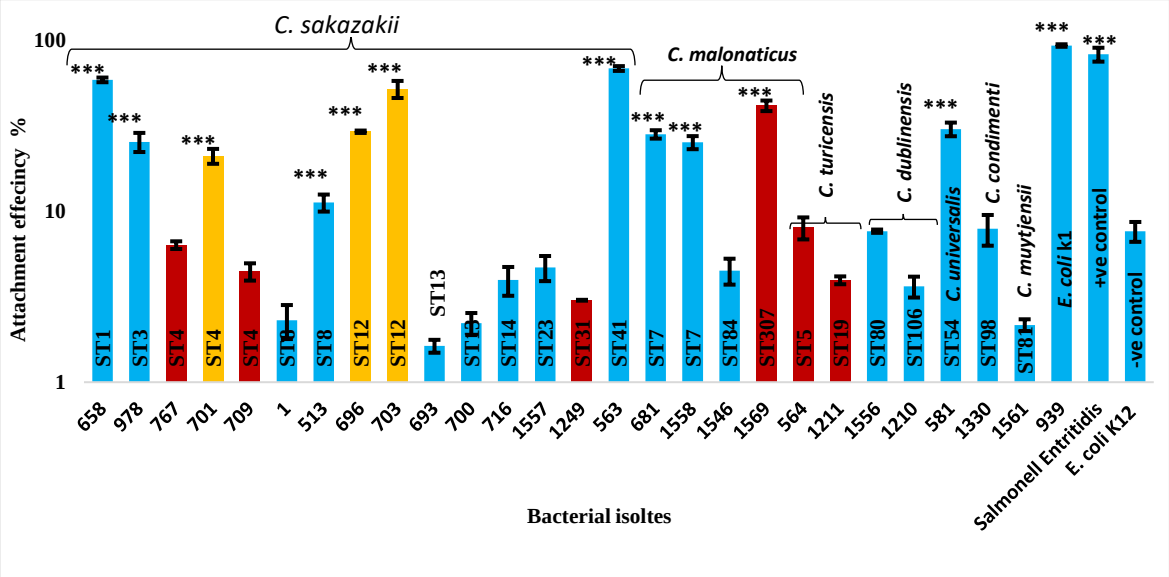


Figure 4-3. Bacterial attachment to H4 cell line after 3-hours incubation at 37°C and 5% CO_2. Results showing the differences in attachment levels among strains. The displayed data are the mean ± standard deviation of attachment efficiency as% of the initial inoculum (MOI~1000) of three independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment and –ve control (***P<0.001, Tukey's). For more information about the strains, see table 2-1.

NEC isolates | Meningitis or blood isolates | Less clinically significant isolates

C. sakazakii ST3 strain 978 which is an enteral feeding tube isolate revealed about 25% attachment. In contrast, *C. sakazakii* ST13 an ST14 isolates demonstrated low level of adhesion and less than 4% of the inoculum. Most of *C. malonaticus* isolates showed high level of adhesion to H4 cells and higher than that revealed by *C. sakazakii* ST4 and ST8

isolates. *C. malonaticus* strains 681, 1558 both belonged to sequence type 7 (ST7) and 1569 ST307 presented 28%, 25% and 41% respectively which considered significantly higher than the non-pathogenic *E. coli* K12 (P<0.001). The adhesion of *C. universalis* strain 581 (ST54) was approximately 30% from the initial inoculum while the rest of *Cronobacter* isolates showed adhesion less than 10%. Interestingly, *the E. coli* K1 NTU ID 939 which is an enteral feeding tube isolate, presented the highest adhesion among of all investigated isolates (P<0.001), which is 93.87% as an average of three independent experiment. This particular group of bacteria is known to be the most responsible for neonatal meningitis among Gram-negative bacteria. All of the adhesion results calculated by subtracting of the invaded bacteria from the number of attached bacteria.

With regard to human epithelial colorectal adenocarcinoma cell line (Caco-2)， bacterial isolates showed different behaviour (Figure 4-4). Most of strains with significant clinical history revealed attachment significantly less than that displayed with H4 cells (P< 0.05) using GraphPad Prism/Paired t test.

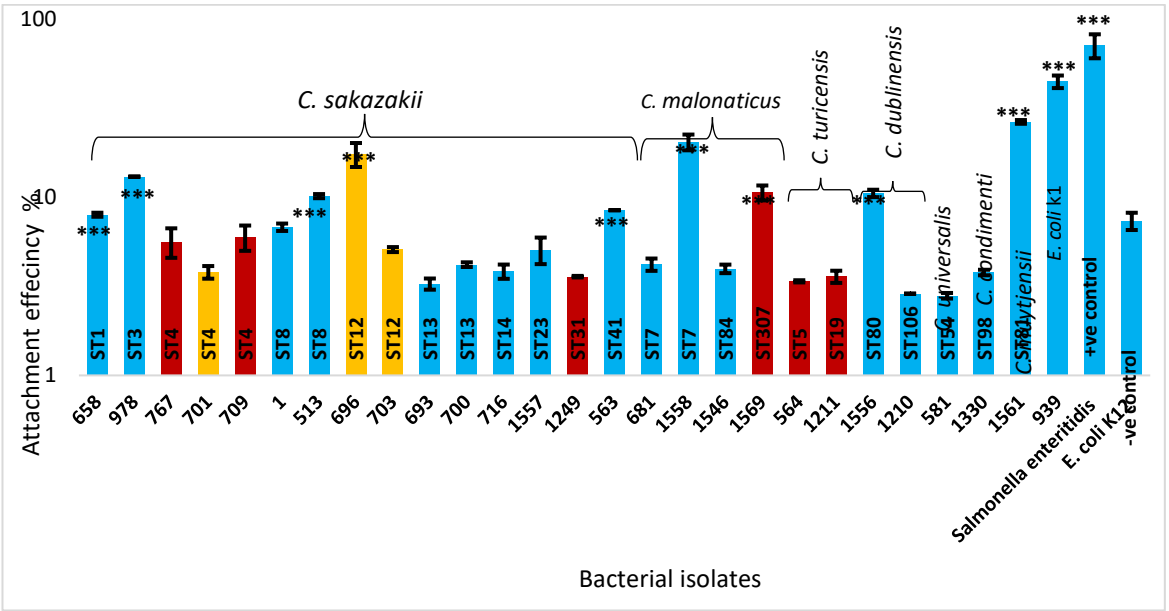


Figure 4-4. Bacterial attachment to Caco-2 cell line after 3-hours incubation at 37°C and 5% CO_2. Results showing the differences in attachment levels among strains. The displayed data are the mean ± standard deviation of attachment efficiency as% of the initial inoculum (MOI~1000) of three independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment and –ve control (***P<0.001, **P<0.01 Tukey's). For more information about the strains, see table 2-1.

For example: French isolate *C. sakazakii* 701 which is associated with a neonatal death due to development of necrotising enterocolitis type 3 (NECIII), showed only 4.5% adhesion compared with 21% of the inoculum attached to H4 neonatal cells. Moreover, *C. sakazakii* 703, and 696 showed 5% and 17% which are about 12 and 2-folds less than that presented with H4 cells respectively. Likewise, *C. sakazakii* strain 563 ST 41 which is foot wound isolate showed 8.4% adhesion which is about 9-fold less than H4 cells. All of *C. malonaticus* isolates showed attachment to H4 cells higher than toCaco-2 cells and up to seven-fold. For example: *C. malonaticus* isolate 681 and ST7 presented 30.6% attachment to H4 compared with 4.8% to Caco-2, and isolate 1569 presented adhesion to Caco-2 cells about 4-fold less than to H4 cells.

Interestingly, this was also observed by the enteral feeding tube isolate *E. coli* K1 strain 939, as it displayed an attachment to H4 cells about two times higher than Caco-2 cells. These results indicate that *C. sakazakii* ST12 strains generally were more adhesive to both cell lines more than any other ST. The attachment process indicates the ability of investigated strains to interact with host cells and potentially invade these cells. Forsythe *et al,* (2014) reported the association of this ST with neonatal NEC, and the high observed adhesion to H4 cells by strain from this ST could be a possible factor in the incidence of NEC.

4.3.2.2 Bacterial Invasion into H4 and Caco-2 cells.

The internalization of bacteria by H4 neonatal intestinal cells was determined after the non-internalized and attached bacterial cells were killed in gentamicin protection assay. This assay was performed to investigate the capability of these strains to enter and survive in human cells. There was no clear correlation observed between invasion and attachment results of the selected strains. *C. sakazakii* ST23 strain 1557 a bronchial secretion isolate, one of the lowest adhesive strains presented the highest level of invasion 1.03% of the inoculum, followed by *C. sakazakii* ST4 strains 701 and 767 showed 0.8% and 0.56% respectively, whichwere all significantly higher than the negative control E. coli K12 ($P<0.001$) as indicated on Figure 4-5.

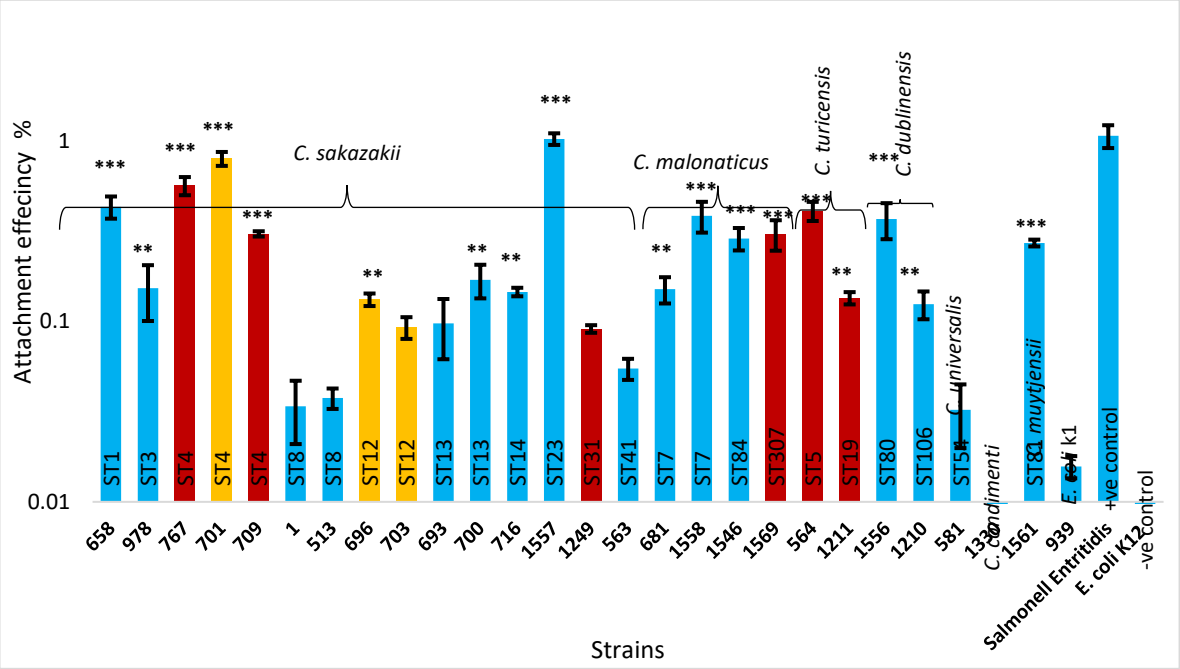


Figure 4-5 Bacterial invasion to H4 cell after 3-hours incubation at 37°C and 5% CO_2. Results showing the differences in invasion levels between strains. The displayed data are the mean ± standard deviation of invasion efficiency as% of the initial inoculum (MOI~1000) of three independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment and –ve control (***P<0.001, **P<0.01- Tukey's). For more information about the strains, see table 2-1.

NEC isolates | Meningitis or blood isolates | Less clinically significant isolates

Other *C. sakazakii* strains ranged between .0 .037% as show by strain 513 to 0.43% by ST1 strain 658. *C. malonaticus* strains 681 and 1558 both from ST7 and invaded H4 with percentage of 0.15% and 0.38%, whereas strain 1569 and 1558 both showed 0.28% invasion into H4 cells. *C. turicensis* isolates 564 and 1211 displayed 0.41% and 0.13% respectively, while *C. dublinensis* type species strain 1210 displayed only 0.12% invasion to H4 cells compared with 0.37% displayed by strain 1556 from same species.

However, *Cronobacter condiment* type species 1330 which was one of the lowest adhesive strains was not able to invade H4 cell lines and displayed the lowest level of invasion among *Cronobacter* strains of 0.006%. *C. muytjensii* strain 1561, which is the type species at NTU collection invaded H4 cells with average of 0.27%. With regard to *C. sakazakii* ST3 isolate 978, which displayed 0.15%, this might be due to the high cytotoxicity observed by this isolate as it found to kill and destroy up to 90% of human cells, and invaded bacteria might be killed during gentamicin protection assay. Unexpectedly, the *E. coli* K1 strain 939 showed only 0.0157% invasion to H4 cells, which

is slightly higher than the negative control *E. coli* K12 although the *Salmonella* isolate showed the highest invasion as a positive control.

Bacterial isolates were also investigated for their invasion into Caco-2 cells. The experimental conditions were similar to that applied for H4 cells, in order to clarify any differences in bacterial invasion to both cell lines and results are shown in figure 4-6.

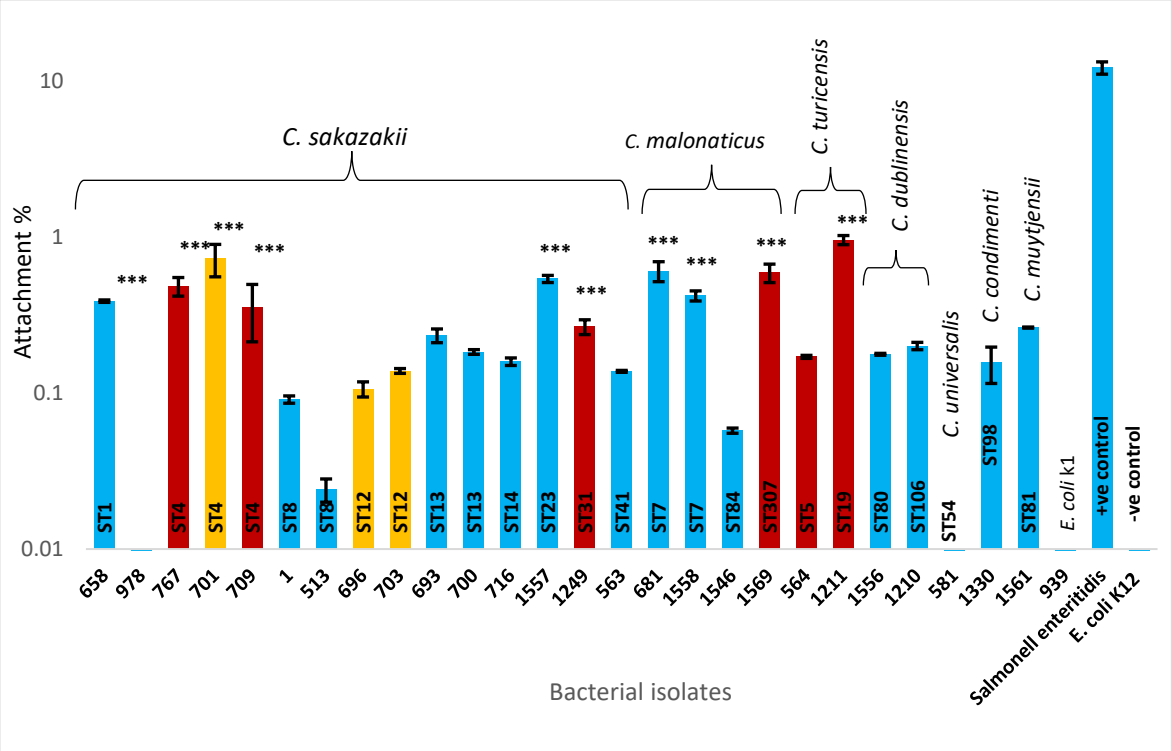


Figure 4-6 Bacterial invasion to Caco-2 cells after 3-hours incubation at 37°C and 5% CO_2. Results showing the differences in invasion levels between strains. The displayed data are the mean ± standard deviation of invasion efficiency as% of the initial inoculum (MOI~1000) of three independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences between the strains in this experiment and –ve control (***P<0.001, **P<0.01 and P<0.05; Tukey's). For more information about the strains, see table 2-1.

In contrast to the results obtained from attachment, when most of pathogenic isolates presented higher attachment to H4 than Caco-2, some strains were slightly more invasive to Caco-2 cells than H4. For example: *C. sakazakii* ST4 strains 767 and 709 invaded Caco-2 with average of about 0.62%, 0.35% compared with 0.56% and 0.30% to H4 cells respectively. However, results obtained by these strains were significantly higher than the negative control *E. coli* K12 (P<0.001) as indicated in Figure 4-6. However, some strains showed less invasion to Caco-2 than H4 cells such as *C. skasakii* ST23 strain

1557 that showed invasion to Caco-2 about 0.52% compared with 1.03% to H4 and also *C. malonaticus* 1546 (ST84) the internalized cells was about 0.057% compared with about 0.4% invasion to H4 cells. The invasion assay determined that most, if not all of investigated isolates were able to invade both cell lines higher than the negative control *E. coli* K12.

4.3.2.3 Patterns of bacterial adhesion with H4 cell line human epithelial cells.

Microscopic examination of infected H4 and Caco-2 cell lines was applied to visualize the pattern of adhesion of selected strains. This was conducted using Giemsa stain techniques as described in section 2.8.5.8.

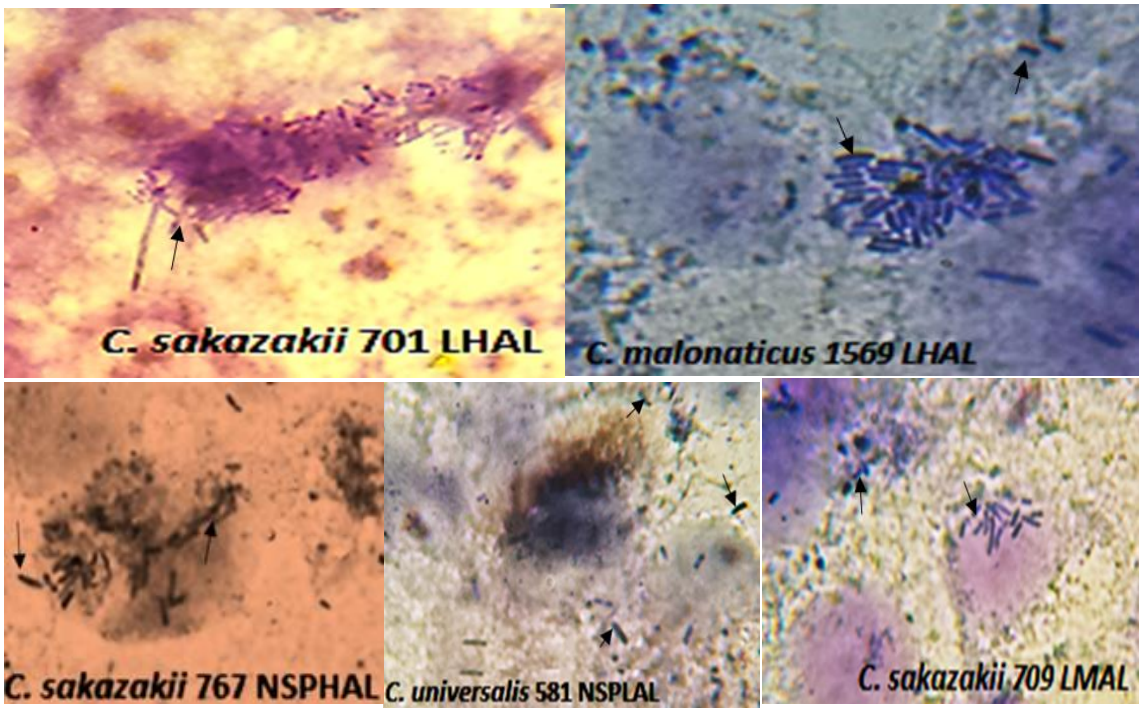


Figure 4-7 Adhesion pattern of bacterial isolates on infected H4 cell line after 3 hour. After infection period, cells were fixed with 100% cold methanol, stained by 7% Giemsa stain, and then examined using oil immersion (100x). Arrows point to bacteria attached to H4 cells. Non-specific pattern high adhesion level (**NSPHA**L) showed by *C. sakazakii* ST4 strain 767 (meningitis), localised high adhesion level (**LHAL**) showed by *C. sakazakii* ST4 strain 701 (NECIII) and *C. malonaticus* ST307 strain 1569 (meningitis), localised moderate adhesion level (**LMAL**) showed by *C. sakazakii* ST4 strain 709 (septicaemia) and non-specific pattern low adhesion level (**NSPLAL**) showed by *C. universalis* ST54 strain 581 (Non-clinical/type species)

Bacterial attachment can be categorised to four distinguishable patterns according to the number and place of adhesion to human cells. These patterns are: Non-specific

pattern High adhesion level (NSPHAL), localised high adhesion level (LHAL), localised moderate adhesion level (LMAL) and non-specific pattern low adhesion level (NSPLAL), and example is shown in figure 4-7 and strains groups are stated in table 4-1.

Table 4-1 Pattern of bacterial adhesion to H4 cells

Pattern of adherence	**NSPLAL**	**LMAL**	**NSPHAL**	**LHAL**
Strain	716, 1, 1330, 581, **1211**[d], 693, 1210, 1561, 513, **564**[b], 1556	**709**[c], 563, **703**[a], **696**[a], 978, 1546	1557, **767**[b],700, 1249[b], 1558	**701**[a], 681, **1569**[b]

NSPHAL: Non-specific pattern High adhesion level, **LHAL**: localised high adhesion level, **LMAL**: localised moderate adhesion level and **NSPLAL**: non-specific pattern low adhesion level**. Bold:** indicating to significant pathology; **a:** NEC strains, **b:** Meningitic strain, **c:** septicaemia and **d**: fatal isolate.

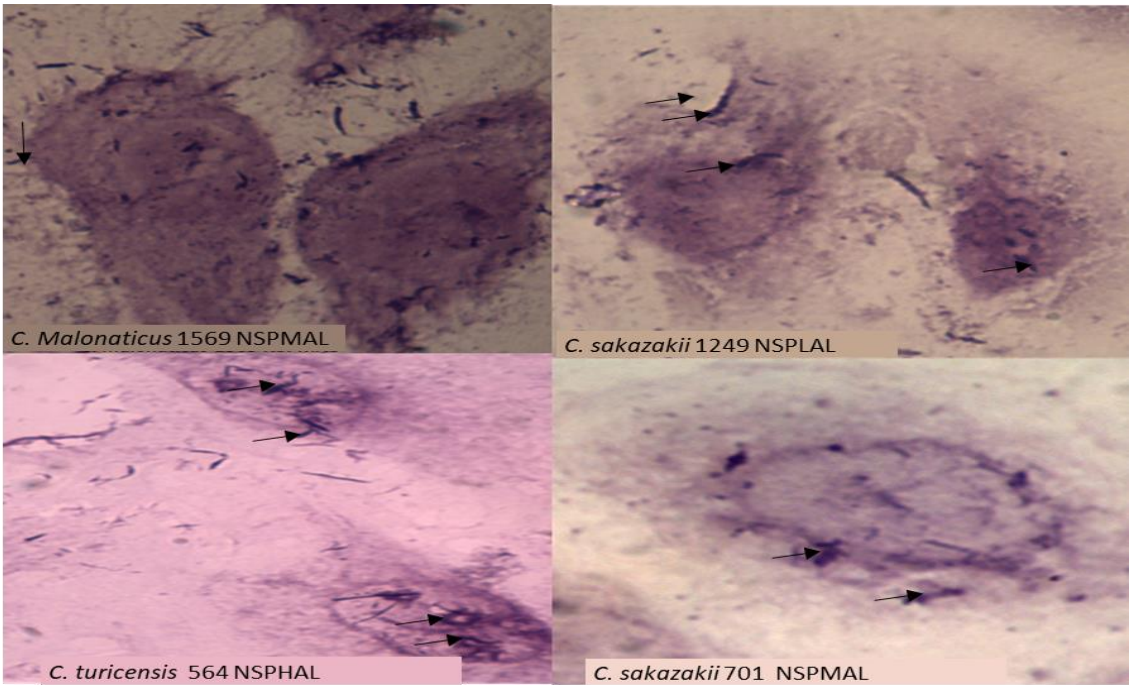


Figure 4-8 Adhesion pattern of bacterial isolates on infected Caco-2 cell line after 3h. After infection period, cells were fixed with 100% cold methanol, stained by 7% Giemsa stain, and then examined using oil immersion (100x). Arrows point to bacteria attached to H4 cells. Non-specific pattern high adhesion level (**NSPHA**L) showed by *C. turicensis* strain 564 (meningitis), localised high adhesion level (**LHAL**) showed by *C. sakazakii* ST4 strain 701 (NECIII) and *C. malonaticus* ST307 strain 1569 (meningitis), localised moderate adhesion level (**LMAL**) showed by *C. sakazakii* ST4 strain 709 (septicaemia) and non-specific pattern low adhesion level (**NSPLAL**) showed by *C. universalis* ST54 strain 581 (Non-clinical/type species)

4.3.3 Bacterial translocation

The pathogenic bacteria vary significantly in their interactions with the host intestinal cells during the process of establishing disease. (Polotsky *et al,* 1994; Siebers *et al,* 1995; Harvey *et al,* 1999). However, in order to cause disease, pathogens have to be able to escape the host defence mechanisms and breach the epithelial cell barriers. The infiltration of epithelial cells by invasive pathogens can occur through many strategies of invasion or translocation (Harvey *et al,* 1999).

Bacterial isolates that are associated with significant pathology and those that revealed significant invasion were further investigated regarding their translocation throughout polarized monolayer of H4 and Caco-2 cell lines. These strains are: *C. sakazakii* strains 693 (ST13) asymptomatic and showed moderate adhesion and invasion, 701 (NECIII),709 (septicaemia) ,767 (meningitis) which are all ST4, 1249 (meningitis) and strain 1557 (bronchial secretion) and *C. turicensis* strains 564 (Neonatal Meningitis) and 1211 (Fatal infant infection/blood isolate) (Joseph *et al,* 2013), and *C. malonaticus* ST4307 strain 1569 (meningitis) (Joseph and Forsythe 2012c), *E. coli* K1 ST 95 strain 939 meningitic classified serotype as a comparative species, 1230 K12 and *Salmonella* Enteritidis 358 as a negative and positive controls respectively

4.3.3.1 Polarisation of monolayer cells

As described in sections 2.8.5.9.1, human cells were seeded at concentration of approximately 1 X 10^6 per ml, and the Trans Epithelia Electric Resistance (TEER) was measured during the polarization period. Polarization of both cell lines was confirmed by Blue dextran assay, as it was very difficult to achieve polarized monolayer H4 cells, which was also demonstrated by Claud *et al,* (2003).

The polarization of H4 cells with DMEM medium was very slow and it takes up to 10 weeks to perform completely polarized monolayer as confirmed by dextran blue assay. Briefly; when detectable change was observed in the TEER of seeded inserts, 200 µl of 10mg/mL dextran blue were added to representative wells and incubated for 2hrs uinder tissue culture conditions. Afterwards, 100 µl was collected from basolateral chamber and OD was measured at 600 nm. There were no differences in the TEER

between inserts seeded with H4 cells and control inserts at the first three weeks, after that TEER was recorded each week. However, polarization was lost when inserts were soaked with DPBS and cells integrity was damaged, and unfortunately, this happen twice, which is in agree with Sanderson et al, (1996) who established this cell line and suggested that it has weak tight junction formation. Therefore, this step repeated again the polarization was confirmed after about 10 weeks, and cells carefully soaked with fresh DMEM instead of DPBS for washing step.

With regard to Caco-2 cells, the polarization was confirmed after 5 weeks when MEM medium was used. Polarization of both cell lines was confirmed by blue dextran assay before using in translocation experiment. Translocation techniques was described in section 2.8.5.9.2, and the results obtained from translocation assay indicated that all of strains were able to translocate both human cells with variable levels. Results presented as a percentage of the inoculum after Miles and Misra method was applied.

4.3.3.2 Translocation assay

All strains revealed more translocation through H4 cell than Caco-2 cells (80%) at 1h one ($P < 0.05$) as shown in the graph Figure 4-9. With regard to H4 cells, the most highly translocated isolate was the meningitic isolate *C. sakazakii* ST4 strain 767 which showed about 0.99% from the initials compared with 0.16% for Caco-2 cells, followed by *C. sakazakii* ST 13 strain 693 which is an asymptomatic isolate and showed translocation by 0.68% compared with 0.22% through Caco-2 cells. However, despite that both strains translocated higher in H4 cells, their translocation through both cell lines was significantly higher than the negative control ($P<0.001$). Moreover, the translocation of *Cronobacter sakazakii* ST4 strains 701 (NECIII) and 709 (septicaemia) were comparatively much lower than strain 767, and displayed more translocation through H4 cells compared with Caco-2 cells. Interestingly, after one-hour incubation all of blood isolates revealed more ability to penetrate the H4 monolayer cell line with range of 6 to 29 folds more than that observed by Caco-2 cells. For example, strains 709, 1249, 564, 1211 and enteral feeding tube isolate *E. coli* strain 939 showed 0.009%, 0.61%, 0.51%, 0.18% and 0.14% translocation throughout H4 compared with 0.001%, 0.16%, 0.017%, 0.037% and 0.002% for respectively.

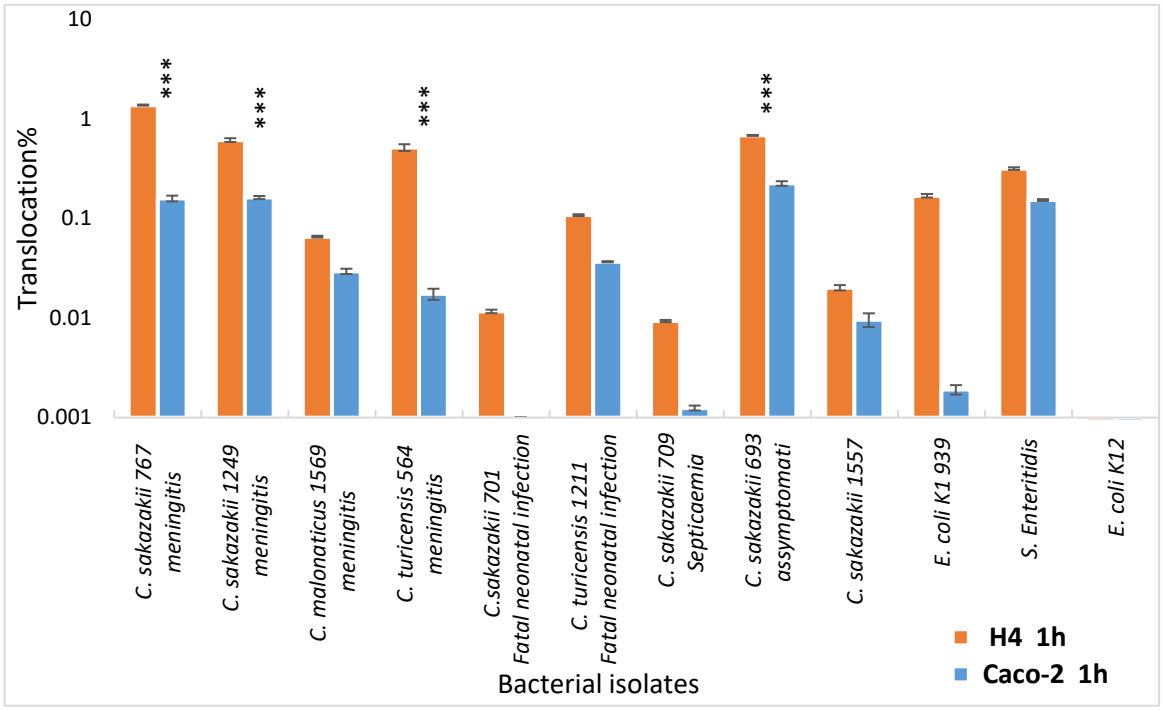


Figure 4-9 Bacterial translocation through H4 and Caco-2 polarized monolayer after 1 hour. Initial inoculum (300 µl) of approximately 10^6/ml of bacteria inoculum were added to the upper chambers, then 100µl collected from the basolateral chambers after 1hand serially diluted in PBS, and Miles and Misra was applied for viable counts. ***Significant (P<0.001).

Negative control was not able to pass through the both cell lines while *S*. Enteritidis the positive control demonstrated 0.26% with H4 compared with 0.153% for Caco-2 cells.

After another two hours' incubation (total three hours), bacterial isolates showed increased translocation, and some strains displayed more translocation over Caco-2 cells than H4 cells (Figure 4-9). This was mainly observed by *C. sakazakii* ST4 strain 767 when the translocation over Caco-2 cells was 1.89% compared with 1.84% throughout H4 cells, and ST31 strain 1249 was 1.95% through Caco-2 cells compared with 1.5% through H4 cells showed. Interestingly, the meningitic isolates *C. malonaticus* strain 1569 and *C. turicensis* strain 564 was the highest infiltrated strains at 3h and showed 4% and 4.7% of the initial respectively. Besides, other strains especially blood isolates or isolated from neonates with septicaemia such as *C. sakazakii* ST4 strain 709 and *C. turicensis* ST 19 strain 1211 (type species of NTU collection) continued to show translocation over H4 cells up to fifty times higher than Caco-2 which noted by *C. sakazakii* ST4 strain 709.

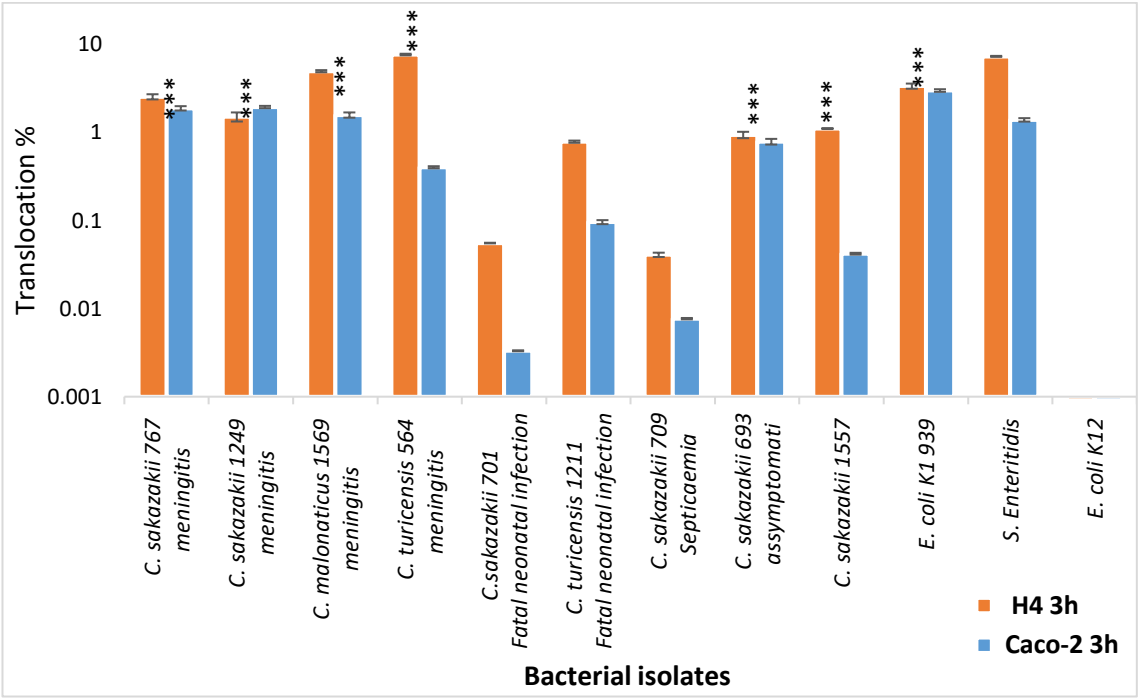


Figure 4-10 Bacterial translocation through H4 and Caco-2 polarized monolayer after 3 hours. Cells after one hour incubation. Initial inoculum (300 µl) of approximately 10^6 were added to the upper chambers, then 100µl collected from the basolateral chambers after 1h and serially diluted in PBS, and Miles and Misra was applied for viable counts. ***Significant (P<0.001).

Moreover, *E. coli* K1 strain 939 displayed more translocation through Caco-2 cells at time two than that recorded with H4, and negative control *E. coli* K12 NTU ID 1230 continued to be non-invasive to both cell lines at 3h.

The effect of bacteria on the polarized H4 cells integrity over three hours incubation was recorded (Figure 4-10), to determine the necrotic effects of bacterial isolates on polarised monolayers. The TEER was recorded before starting the experiment and at time point 1(1hr) and time point 2 (3h). After 1-hour incubation minor decrease was observed by most strains, while some strains such as *C. sakazakii* ST4 strain 701 and *C. turicensis* ST5 strain 564 the TEER was slightly increased, which might be because of gathering and attaching of bacterial cells to H4 cells. This increase was also noted for non-invasive *E. coli* K12 the negative control. However, inserts infected with *C. sakazakii* ST4 isolate 767 showed a drop in the TEER at time one from 275 Ohms to 235 Ohms/cm^2. After another two hours, there was a sharp drop in the TEER of some inserts, particularly clinically significant strains which also showed high translocation at time point two. The NECIII isolate *C. sakazakii* ST4 strain 701 showed a slight increase in TEER after 1 hr, after another two hours the TEER was sharply decreased from 277 Ohms/cm^2 to 210 Ohms.

Furthermore, *C. sakazakii* meningitic isolates 767 and 1249 damaged the integrity of the polarized monolayer and resulted in loss of the TEER to 185 and 187 Ohms/cm^2 from 235 and 255 Ohms/cm^2 respectively. *E. coli* K1 also caused TEER reduction from 267 Ohms/cm^2 at 1he to 167 Ohms/cm^2 at 3h. This in interesting way is supporting the translocation assay results.

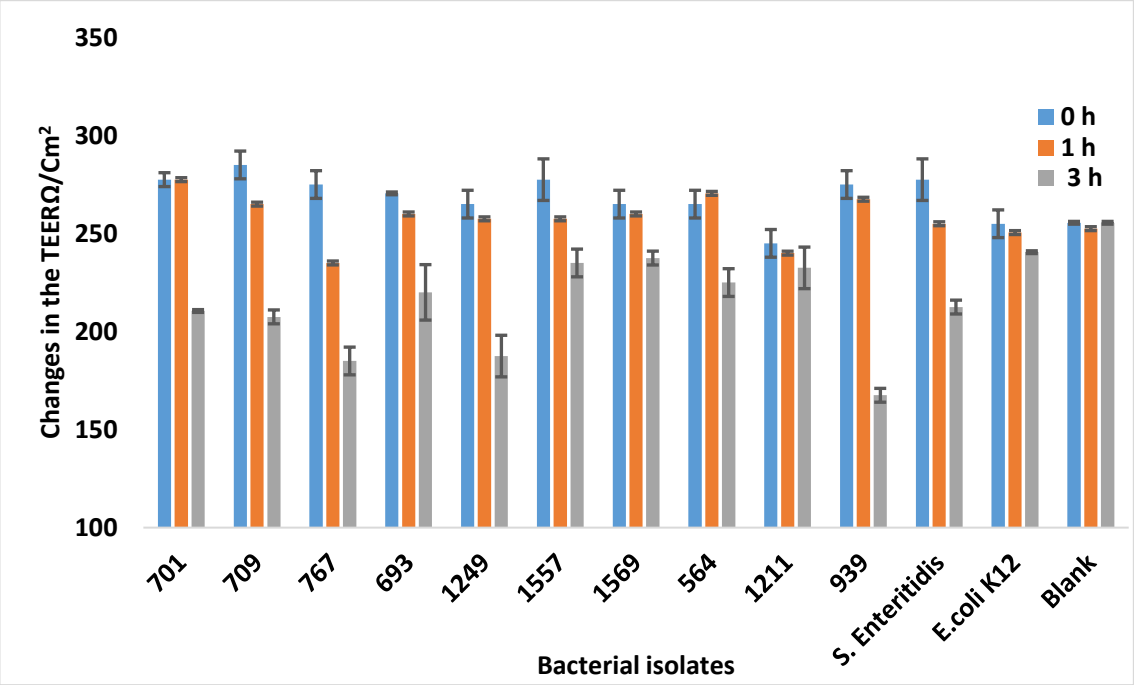


Figure 4-11 Changes in the TEER of H4 polarized monolayers over 3h incubation. Bacterial isolates, showing the reduction of the electrical resistance in Ohms over 3h incubation. 0 h is the TEER measurement before adding bacteria to the inserts.

With respect to Caco-2 cells, all strains showed a minor decrease in the polarity as confirmed by TEER measurement compared with H4. Among all strains, only *E. coli* K1 showed 20 Ohms/cm^2 reduction in the TEER after one hour incubation (Figure 4-11). However, at the end of experiment, few a *Cronobacter* strains showed reduction of more than 30 Ohms/cm^2 of the starting reading which are*: C. sakazakii* 1249, *C. malonaticus* 569 and *C. turicensis* 564 and all of them are neonatal blood isolates with meningitis. Interestingly, this was also recorded by *E. coli* K1 the serotype linked with neonatal meningitis, and the TEER dropped from Ohms/cm^2 at time two to Ohms/cm^2 at time three and total of 120 Ohms/cm^2 from the starting reading. in contrast, the *C. sakazakii* ST4 strain 701 caused 5 Ohms/cm^2 increase at the end of experiment, and 15 Ohms/cm^2 increase was recorded by negative control *E. coli* K12. The positive control *Salmonella*

Enteritidis, however also showed a major decrease in Caco-2 monolayer polarity and TEER was reduced by about 100 ohms compared with starting polarity.

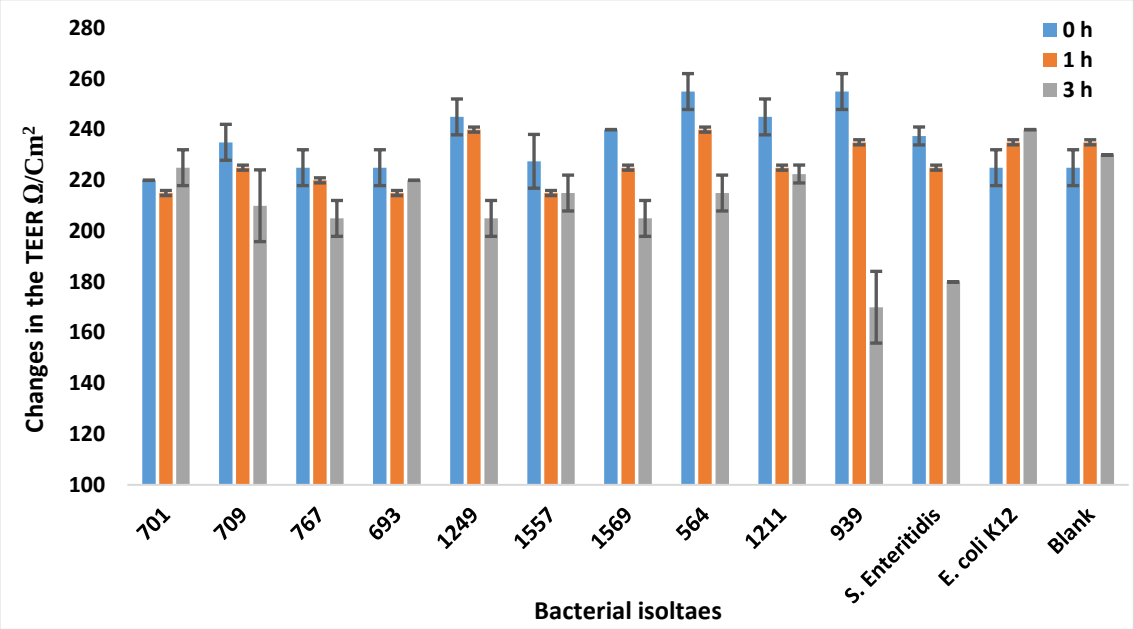


Figure 4-12 Changes in the TEER of Caco-2 polarized monolayers over 3h incubation. Bacterial isolates, showing the reduction of the electrical resistance in Ohms over three hours incubation. 0 h is the TEER measurement before adding bacteria to the inserts.

4.3.4 Genes associated with bacterial adhesion and invasion to host cells

To establish success infection, pathogenic bacteria are required to attach to the host cells, invade, persist, multiply and disseminate to different tissues. This process requires different mechanisms regulated by several genes in the pathogens and/or host cell. Some pathogenic bacteria use macromolecular strictures such as type IV pili and type III secretion systems to establish host pathogen interaction or relationship. All strains were found to be positive for IV pili gene except 1558 *C. malonaticus,* yet none of the investigated strains have *eprh* gene responsible for type III secretion protein. Hartmann *et al,* (2010) indicated that the flagella is important in biofilm formation and adhesion to Caco-2 intestinal epithelial cells, therefore, strains were screened for flagellum genes *fli* (ESA_01248 - ESA_01261) and *flg* (ESA_02264 -ESA_02277) and found to be present in all strains except *C. turicensis* strain 1211 and 564 *and C. universalis* satrain 581 which do not have *fliI* (flagellum-specific ATP synthase ESA_01257) and *fliG* (Flagellar motor switch protein ESA_01259), while *C. condimenti* was missing only *flgE* (Flagellar hook protein ESA_02271). Moreover, Choi *et al,* (2014) demonstrated that the putative *mcp*

which is encoded in pCSA2 is important for adhesion and invasion. This gene is absent in all investigated strains except *C. sakazakii* ST8 type species strain 1. Strains were further searched for genes responsible for a hypothetical protein that was found to effect bacterial adhesion Caco-2 cells (Hartmann *et al,* 2010)

Invasion to host cells was found to be controlled by different genes located on pathogen's genome such as in *E. coli* and *Salmonella* which differ in their functions (Farr *et al,* 1989, Badger *et al,* 2000, Bessman *et al,* 2001, Ismail *et al,* 2003, Peng *et al,* 2012). The invasion of *E. coli* and *Salmonella typhimurium* to human epithelial cells was promoted by the expression of *apaH,* and *ompA* is required for *Cronobacter* invasion of human brain microvascular epithelial cells (HBMECs) and is associated with neonatal *C. sakazakii* meningitis (Mohan Mohan Nair and Venkitanarayanan 2006; Singamsetty *et al,* 2008; Mittal *et al,* (2009)).

Moreover, invasion of *E. coli* K1 to HBMEC was associated with present of *ompX*, *ompA*, *ppk1*, and *ygdP* (Badger *et al,* 2000, Singamsetty *et al,* 2008, Mohan Nair *et al,* 2009, Kim *et al,* 2010, Peng *et al,* 2012). OmpX and OmpA have been reported to be important for the basolateral invasion of some mammalian cells such as spleen and liver of rat pups (Kim *et al,* 2010a)

Strains were investigated for the presence of the invasion-associated genes *apaH, ompA, ompX, ygdP, and ppk1* using *Cronobacter* MLST BLAST database. Although, strains were varied in their invasion to both cell lines, they all carried these genes, which proposing that the expression of these genes might differ, or different genes/host components are involved in this process

4.3.5 Serum resistance.

After pathogenic bacteria success in invasion and penetrating of the epithelial barrier, it has to be able evade host defences agent such as macrophages and serum bactericidal activities. Schwizer *et al,* (2013) demonstrated that *C. sakazakii* showed the highest serum tolerance in a study which included different species of genus *Cronobacter.*

In this study, 26 bacterial isolates from the 7 species of *Cronobacter,* and one *E. coli* K1 isolate as well as the positive control *Salmonella* Enteritidis and negative control *E. coli* K12 were exposed to serum as described in section 2.8.4.11.

The investigated strains showed variable levels of resistance to serum bactericidal activity, which is a potential factor in the pathogenicity of this genus. Some strains showed an increase of bacterial cells number up to 150% ($P<0.001$) such as the meningitic *C. sakazakii* ST31 strain 1249, and about 105% increase was obtained by *C. sakazakii* ST4 strain 709 that was associated with neonatal septicaemia. More interestingly, the highest recorded survival was obtained by *C. sakazakii* isolates and mainly by blood isolate which are ST4 709 and ST31 1249. Only two (13%) of *C. sakazakii* isolates were sensitive to serum killing which are 696 ST12 and 716 ST14 (Figure 4-13). With regard to *C. malonaticus* isolates 75% (3/4) showed moderate resistance to serum activity, while strain 681 which is the type species at NTU collection was sensitive to serum.

Furthermore, *C. turicensis* strain 564 ST5 which is originally blood isolate from neonate suffering of severe meningitis, showed a moderate level of serum resistance which is was also recorded by strain 1211 (NTU type species. *C. dublinensis* type species isolates strain 1210 (non-clinical isolate) was sensitive while the clinical isolate 1556 ST88 showed the highest resist among the non *C. sakazakii* isolates. The other two *Cronobacter* type species *C. condimenti* and *C. muytgensii* can be considered as serum sensitive strains as both showed less than 0.008% recovery after three hours' incubation.

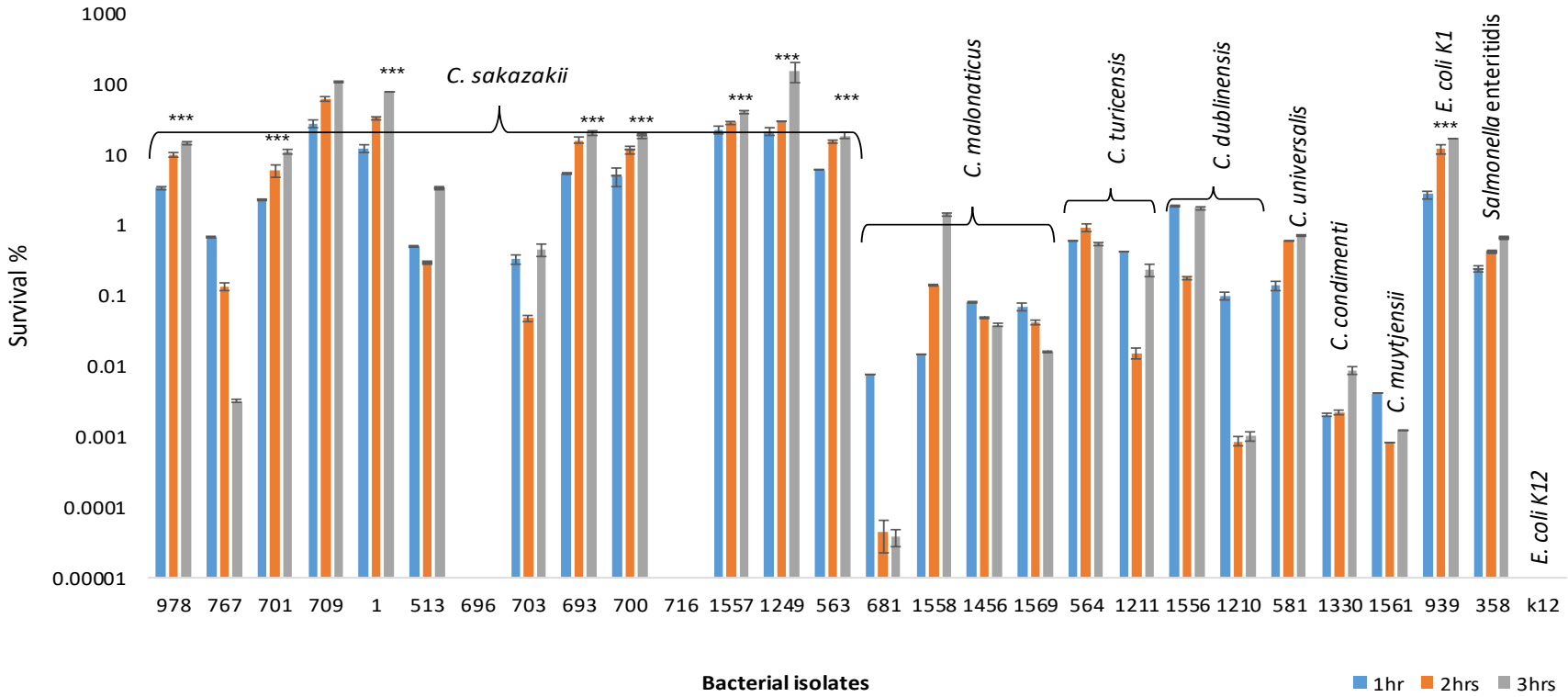


Figure 4-13 Sensitivity of bacterial isolates to human serum over 3 hours of incubation showing the difference in % survival among strains over 3h time. Most of the strains showed increases in their viable counts including *S.* Enteritidis, and strains 70 and 709 (ST4), 693 (ST8), and 1249 (ST31), whereas *C. sakazakii* 696 (SR12), 716 (ST14) and K12 showed significantly reduced viability. The displayed data are the mean±standard deviation of survival% (10^7 cfu/ml initial inoculum) compared to time 0 of two independent experiments. The asterisks above the bars indicate statistically significant differences between bacterial isolates and negative control *E. coli* K12 strains in this experiment (*P<0.001; Tukey's test). Although there was a significant differences between strains and *E.coli* k12 and strains each other's at all time points, asterisks indicated to only significant differences at 3h as the final result of the experiment.

K1 strain 939 showed an increase in bacterial growth (CFU) from time one (Figure 4-12) 1hto 3h from 2.7% to about 17% of the inoculum (P<0.001). *Salmonella* Enteritidis showed only 0.6% survival, while no growth was detected the negative control (k 12).

4.3.5.1 Genes responsible for serum resistance

A BLAST search of *cpa* (Gene bank number ESA_pESA3p05434) was performed for the selected strains (n=26) and results indicated that not all investigated strains possess this gene. Among *C. sakazakii* isolates, only ST8 isolates the type species strain 1 and strain 513 were negative. In addition, all of *C. malonaticus* strains (681, 1546, 1558 and 1569), *C. turicensis* strains 564 and 1211, *C. condimenti* strain 1210 and 1556 and *C. dublinensis* strain were negative for *cpa* gene.

Colanic acid is found to be regulated by *rcsA* gene (Gottesman *et al,* 1985, Allen *et al,* 1987). BLASTA search indicated that all strains are positive for this gene and other colanic acid genes and no correlation between genomic and physiological traits were found.

Strains were also positive for CSK29544_RS18160 OmpA, and CSK29544_RS05485 *OmpA* family lipoprotein of *C. sakazakii,* and outer membrane protein A (OmpA) of *C. turicensis*. However, when strains were searched for CSK29544_RS16375 OmpA-family membrane protein (*C. sakazakii*), the presence of this gene was varied and no specification was noted as it was absent in most of *Cronobacter sakazakii* isolates (12/18) form different STs (658, 513, 1, 767, 693, 696, 700, 701, 703, 709, 1249 and 730). Moreover, CSK29544_RS16375 OmpA-family was missing from *C. malonaticus* strains ST7 strains only (681 and 1558), and *C. universalis* 581 while other strains have harboured this gene. Note: genes sequence was obtained from NCBI website.

Strains were also searched for the hypothetical protein (ESA_04103) gene that was described by Schwizer *et al,* (2013), and only *C. sakazakii* isolates were positive for this gene. This might support the finding of Schwizer *et al,* (2013) as the experimental results indicated that *C. sakazakii* isolates showed highest serum resistance. However, this gene might not be expressed in the two sensitive *C. sakazakii* strains 696 and 716 that were completely sensitive. Furthermore, a study by Phan *et al,* (2013) indicated that there is an association between the murein lipoprotein *lpp* gene and serum resistance in *E. coli.*

Different *lpp* homologous genes from Enterobacteriaceae bacterium strain FGI 57 were obtained from NCBI and searched in the tested strains. All *Cronobacter* were positive for the searched *lpp* genes. Moreover, strains were searched for the genes described by Du *et al,* (2016) related to adhesion and invasion using *Cronobacter* MLST BLAST Databases. Results indicated that *recB* and *fliR* was present in all strains while other genes were variable. The *bglF* gene was absent in all of *Cronobacter* isolates. Only *C. sakazakii* ST8 strain 1 was missing the *fnr* gene, while *flhA* was present in all strains except *C. universalis*. The hypothetical protein encoded gene *(hp*-2a, ESA_04202) were only absent from *C. sakazakii* ST13 strains, while *hp*-3a, ESA_00132 was absent in *C. sakazakii* strain 513 and positive in other strains.

However, there is no strong correlation between the searched genes and experimental results, as an example *cpa* gene was absent in two *C. sakazakii* strains (1, 513) which both showed an increase in the bacterial number after three hours incubation in 50% (v/v) of human serum.

4.3.6 Cytotoxicity of bacterial isolates to H4 cell line compared with Caco-2 cells

4.3.6.1 Cytotoxicity to H4 cells

Cytotoxic activity of the selected bacterial isolates to H4 cell line was examined by co-incubation of human cells with bacteria for a period of 3 hours. Most of the investigated isolates caused an increase in dead H4 cells compared with control, and some strains revealed a very high cytotoxicity levels, for instance, *Cronobacter sakazakii* strain 978 sequence type 3 (ST3) showing greater cytotoxicity in H4 cells than other strains despite no link to disease. Results presented as fold of the blank, this strain revealed more than 92 folds, about 4 times higher than cytotoxicity showed by *S. enteritidis* (figure 4-14).

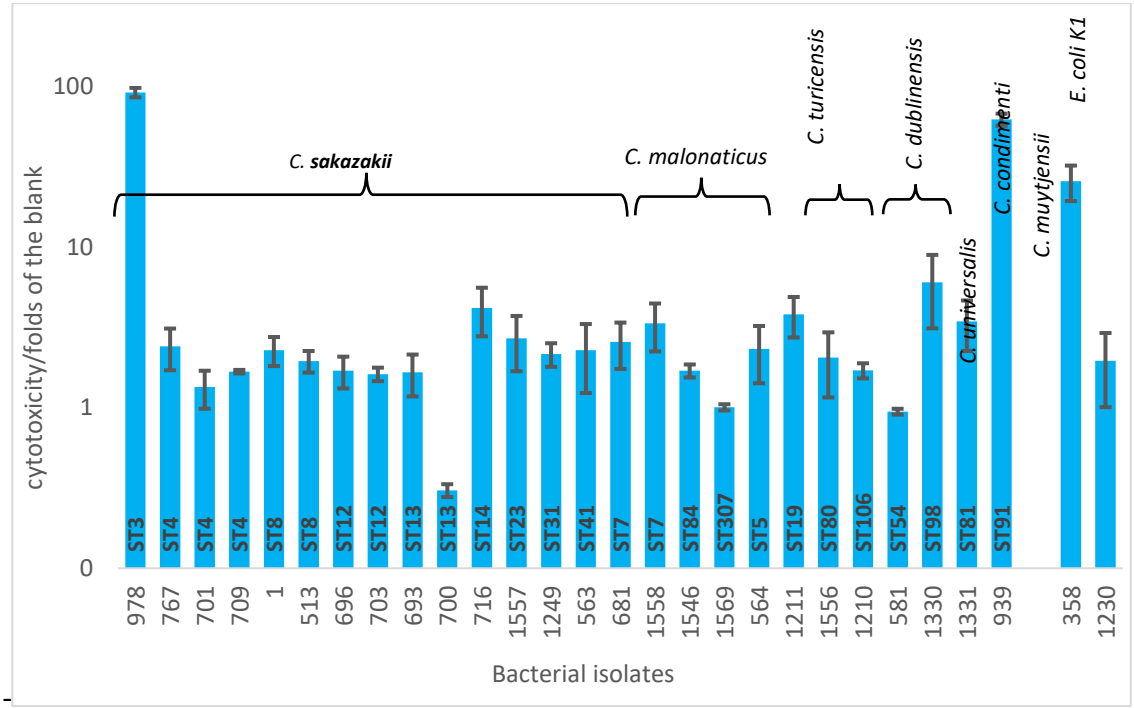


Figure 4-14 Cytotoxicity of selected bacterial strains on H4 up to 3 hours of incubation. Trypan blue cell viability assay was used to measure the cytotoxicity levels of bacterial strains as the dead cells will absorb the Trypan blue dye and be coloured blue while the live cells appear bright. Cytotoxicity calculated as the number of dead cells/total counted cells X 100 and plotted fold increase compared to the blank which only culture medium added to the cells. The data presented in mean±standard error of mean of three independent experiments.

Strain 1330 showed cytotoxicity of about six-fold higher than the blank followed by *C. sakazakii* ST14 strain 716, while *C. malonaticus* strains 700 and *C. universalis* strain 581 did not show any cytotoxicity activity toward H4 cell line. Interestingly, the *E. coli* K1 ST91 strain 939 was also vey cytotoxic towards H4 cells as shown about 62 folds more cytotoxic than the blank (no bacteria added). This particular serotype is commonly associated with neonatal meningitis and high rate of mortality and morbidity (Nudelman and Tunkel, 2009).

4.3.6.2 Cytotoxicity of bacterial isolates to Caco-2 cell line

Cytotoxicity of the selected bacterial isolates to Caco-2 cells was also examined in similar conditions applied for H4 cell line (3h incubation with bacterial isolates at initial concentration of OD_{600} of 0.05). *Cronobacter sakazakii* ST 3 strain 978 was the most cytotoxic strain to this cell line with about 70-folds of the non-treated cells, followed by *C. sakazakii* strain 703 with 6-folds and *C. malonaticus* ST307 strain 1569 which revealed about 4.5 folds of the blank (Figure 4-15). The remaining strains ranged from nontoxic

effects to about fourfold of the blank when only culture medium added to the cells. Likewise,, *E. coli* K1 strain 939 showed high cytotoxic to this cell line and resulted in cell deaths about 62 times higher than the blank. *Salmonella* Enteritidis presented only 7-folds of blank, which considerably high level of cytotoxicity.

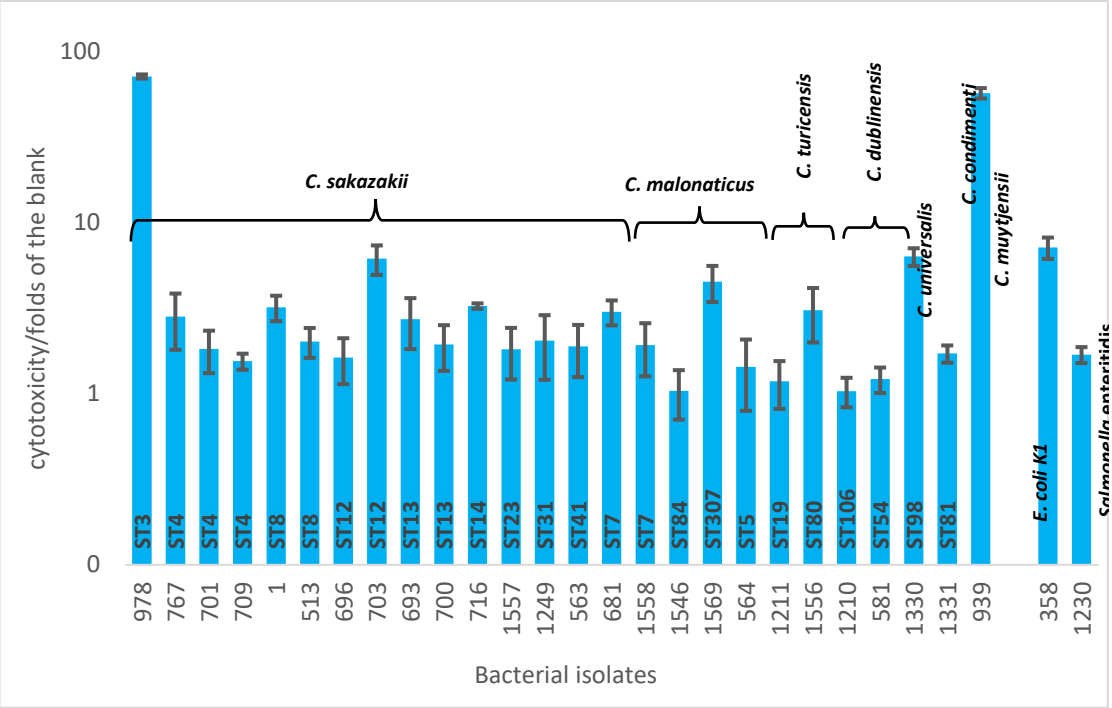


Figure 4-15. Cytotoxicity of selected bacterial strains on Caco-2 over 3 hours of incubation. Trypan blue cell viability assay was used to measure the cytotoxicity levels of bacterial strains as the dead cells will absorb the Trypan blue dye and be coloured blue while the live cells appear bright. Cytotoxicity calculated as the number of dead cells/total counted cells X 100 and plotted fold increase compared to the blank which only culture medium added to the cells. The data presented in mean±standard error of mean of three independent experiments.

4.3.6.3 Cytotoxicity of *C. sakazakii* ST3 isolates

Selected strains from same ST3 group (n=6) were tested for their cytotoxicity for H4 and Caco-2 cells in order to compare with the high cytotoxic isolate, and results indicated that they did not show this level and the cytotoxicity was similar to that obtained from other *Cronobacter* strains (Figure 4.15).

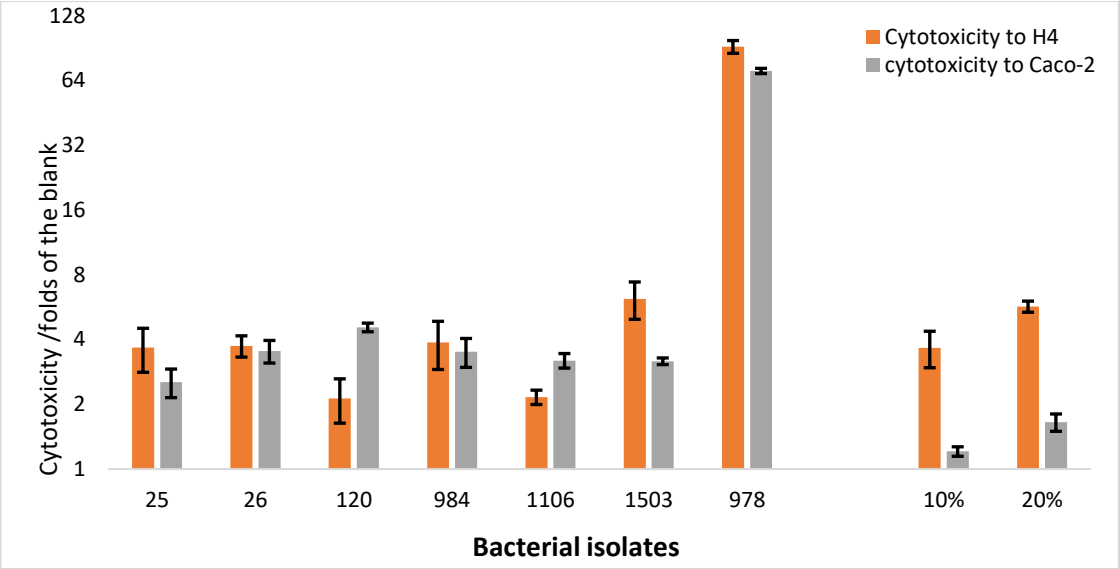


Figure 4-16 Cytotoxicity of *C. sakazakii* ST3 isolates to H4 and Caco-2 cells over 3h incubation. Trypan blue cell viability assay was used to measure the cytotoxicity levels of bacterial strains as the dead cells will absorb the Trypan blue dye and be coloured blue while the live cells appear bright. Cytotoxicity calculated as the number of dead cells/total counted cells X 100 and plotted fold increase compared to the blank which only culture medium added to the cells. The data presented in mean±standard error of mean of three independent experiments. 10% and 20% (v/v) of 16-24h broth culture were added to tissue culture medium and incubated for 3h, and the cytotoxicity tested using Trypan blue as indicated above.

However, most of the selected strains were slightly more cytotoxic to H4 than Caco-2 (P= 0.3355/ paired t test). Interestingly, when 10% and 20% (v/v) of overnight culture filtrate (using 0.22 µm pore size syringe filter) of *C. sakazakii* strain 978 strain were added to appropriate tissue medium, the cytotoxic was up to 6 times higher than the blank, but however no cytotoxic activity was observed of filtrate obtained from 5h growth in both TSB or DMEM medium.

4.3.6.4 Lactate dehydrogenase cytotoxicity assay

The cellular enzyme lactate dehydrogenase (LDH) is widely used as a cell death marker in experimental cytotoxicity assays based on the measurement of changes in membrane integrity associated with cell death. As described in section (2.8.5.11.1), human cells exposed to 3h infection with the selected bacterial isolates and LDH protocol was carried out according to the suppliers recommendations (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega Co.). Cytotoxicity was then calculated as a percentage of maximum leakage control obtained from lysed cells (positive control). Most of the

strains were able to show detectable amounts of LDH leakage from damaged cells due to destruction of the human cell membrane and loss of integrity.

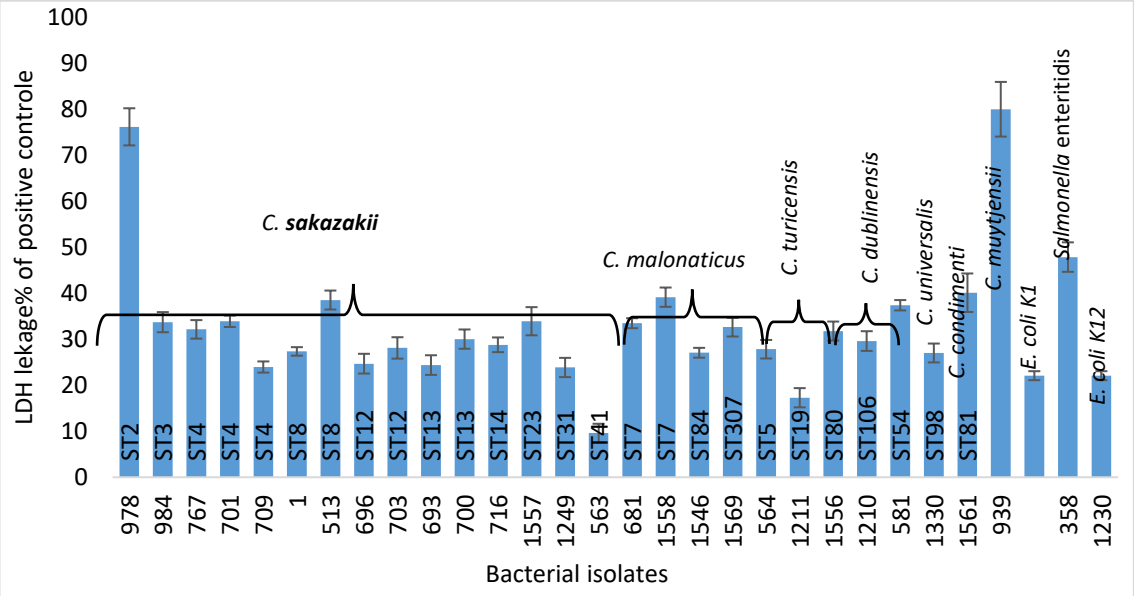


Figure 4-17 leakage of lactate dehydrogenase (LDH) cytotoxicity assay of H4 cells. The increase in LDH was used as indicator of cytotoxic activity of bacterial strains due to loss of cell membrane integrity and outflow of LDH to the growing medium. Cytotoxicity was calculated as percentage of leaked LDH from cells treated with bacteria compared to lysed cells (positive control). The data presented in mean±standard error of two independent experiments.

However, the obtained results from LDH assay supported the cell viability assay using Trypan blue dye, and *C. sakazakii* ST3 strain 978 revealed about 76% cytotoxicity of the total incubated cells after three hours' incubation (Figure 4-17). The *C. sakazakii* ST3 strain 984 which was also isolated from neonatal enteral feeding tube on the same collection day, was also examined and the results was similar to non ST3 isolates. Similarly, K1 strain 939 was the most cytotoxic strain and resulted in about 80% cytotoxicity as indicated by LDH leakage.

With respect to Caco-2 cells, *C. sakazakii* ST3 strain 978 and K1 strain were the most cytotoxic isolate for this cell line and revealed 60% and 62% respectively. Although most of the *C. sakazakii* strains showed same manner with both cell lines, they revealed less cytotoxic behaviour towards Caco-2 than that observed with H4 cells (significantly different $P < 0.05$). For example, the leaked LDH caused by *C. sakazakii* ST8 strain 513 was the highest in both cell lines which is 38% and 26% followed by *C. sakazakii* ST23 strain 1557 that showed 33% and 22% for H4 and Caco-2 cells respectively (Figure 4-18).

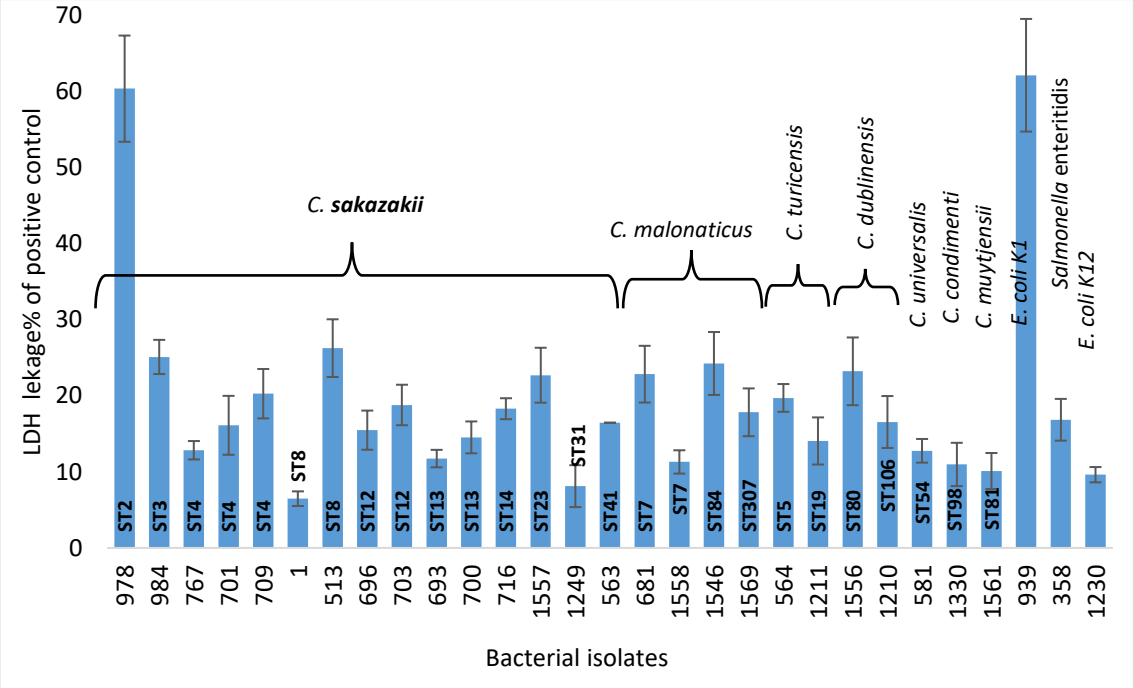


Figure 4-18 leakage of lactate dehydrogenase (LDH) cytotoxicity assay of Caco-2 cells. The increase in LDH was used as indicator of cytotoxic activity of bacterial strains due to loss of cell membrane integrity and outflow of LDH to the growing medium. Cytotoxicity was calculated as percentage of leaked LDH from cells treated with bacteria compared to lysed cells (positive control). The data presented in mean±standard error of two independent experiments.

4.3.6.5 Investigation of potential cytotoxicity of bacterial outer membrane proteins to human cells using Lactate Dehydrogenase (LDH) assay.

The aim of this assay was to investigate the potential role of bacterial outer membrane proteins to cause disease through damaging the host cells. Bacterial outer membrane proteins extracted as described in section (2.8.4.13.2) and then a final concentration of 100µg/ml in appropriate tissue culture medium added to both cell lines for three hours. Results indicated that most of OMPs showed considerable levels of cytotoxicity after the blank subtracted from the obtained OD, and OMPs was obviously more cytotoxic to H4 cells more than Caco-2. Outer membrane proteins from *C. sakazakii* ST 41 strain 1249 was the most cytotoxic to H4 cells (26%) among the investigated strains while OMPs of *C. sakazakii* ST3 strain 978 was the highest cytotoxic to Caco-2 cells and showed about 7% cytotoxicity. Interestingly, OMPs from *C. sakazakii* ST3 strain 978 were more cytotoxic to H4 cells than OMPs of the cytotoxic isolate 984 from same ST group.

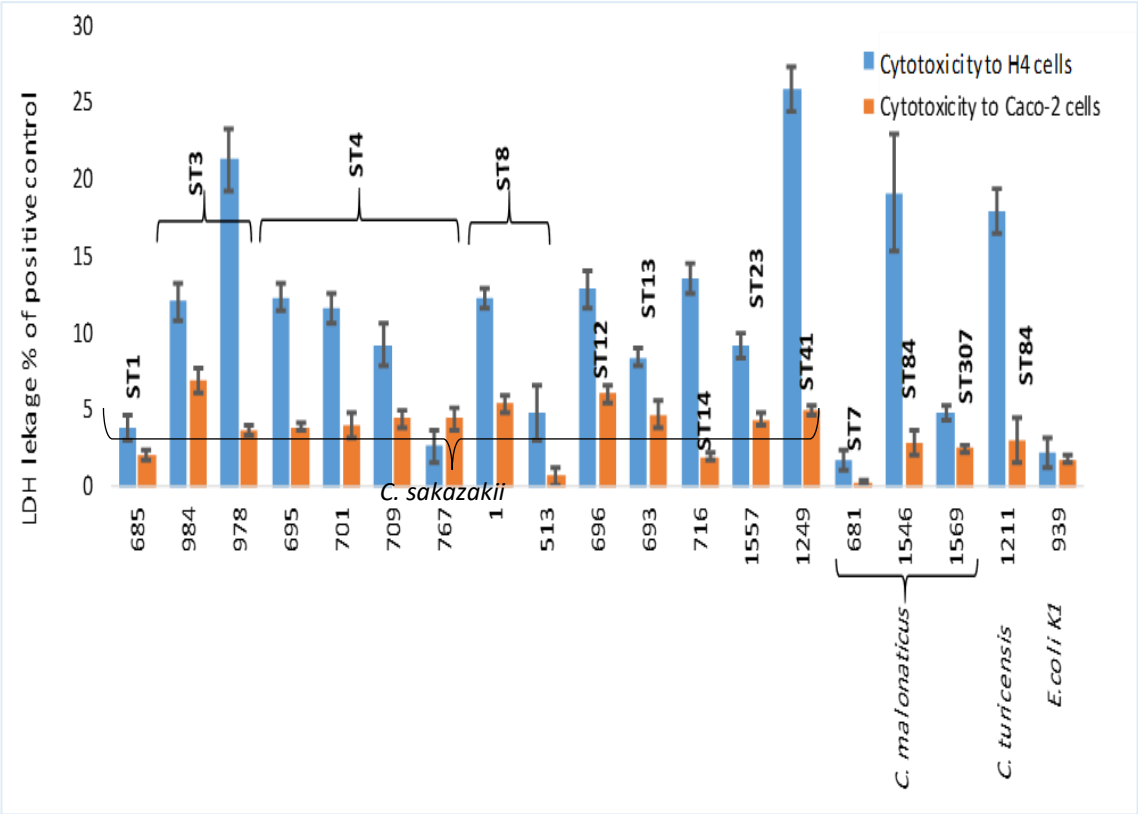


Figure 4-19 . Cytotoxicity of OMPs from selected bacterial isolates to H4 and Caco-2 cells. Human cells were exposed to 100 µg/ml of bacterial OMPs for 3h and then cytotoxicity was measured using lactate dehydrogenase assay. The displayed data are the mean±standard deviation of cytotoxicity% of the maximum lactate dehydrogenase cytotoxicity of two independent experiments in triplicate

Moreover, OMPs of *C. malonaticus* strain 1546 and *C. turicensis* strain 1211 both showed high level of cytotoxicity compared with control when no OMPs were added to the cells. Interestingly, when commercial lipopolysaccharides from *E. coli* used in this assay as a comparative control, it showed the highest cytotoxic effects to both cell lines. In general, this assay indicated that OMPs separate of live organism may play role in host cells proliferation and increase cells death, which might aide bacterial invasion and translocation to underlying layers.

4.3.6.6 Cytotoxicity related genes.

Limited data are available about the mechanisms involved in *Cronobacter* cytotoxicity or association of specific genes with the cytotoxic activity of this genus (Kucerova *et al,* 2010; Tall *et al,* 2014). *Cronobacter* isolates were screened for genes that were potentially associated with cytotoxicity to host cells and/or genes responsible for

haemolytic enzymes. *HlyA* gene encodes for α-haemolysin that can degrade varied range of host cell types (Dhakal and Mulvey, 2012). All strains except *C. condimenti* 1330 were positive for the putative membrane protein insertion efficiency factor *hlyA* gene, while *hlyA* hemolysin (ESA_RS01960) encoded for type III hemolysin and described by (Cruz *et al,* 2011) was presented in all investigated strains.

Furthermore, strains were searched for the *YkfI* gene encoding for toxic protein that inhibits cell growth, decreases viability and induces immediate cell lysis (Yan *et al,* 2013; Wen *et al,* 2017). Two different genes were found in the NCBI database with this name which are *ykfI* (CSK29544_RS21270) 342 nucleotides, was messing in most strains (62%) and presented in *C. sakazakii* strains 513, 1, 696, 703 and *C. malonaticus* strains 681, 1546, 1569, *C. condimenti* 1330 *C. dublinensi* strains 1210, 1556. The other *ykfI* gene is (CSK29544_RS10295) 327 nucleotides, and present in *C. sakazakii* strains 1, 696 and 703 and 513, in *C. malonaticus* strains and 681, 1546 and 1569, as well as *C. dublinensis* 1556. The remaining strains (66%) were negative for this gene. These to genes are differ in about 15 nuclides. Moreover, *tisB* gene encodes for a toxic inner membrane peptide (Weel-Sneve *et al,* 2013) and was present in all investigated *Cronobacter* isolates.

4.3.6.7 Investigation of ST3 isolate 978

The high level of cytotoxic behaviour of *C. sakazakii* ST3 isolate 978 was of interest and investigated further. The first step applied to investigate the cytotoxic activity of this strain was incubation of human cells with filtrate of 3h growth in DMEM medium. The pH was adjusted to normal level using 0.1 M filter-sterilized sodium hydroxide (NaOH), and then added to human cells for 3h, and cell viability was taken. Results indicated that this filtrate does not kill human cells and no differences were recorded between normal medium (control) and filtrate, which is in contrast of the adding of 10% and 20% filtrate of overnight bacterial broth culture to DMEM that showed about 6-folds cytotoxicity. This directed the research to new experiments as the presence of bacterial cells appeared to be key factor of its cytotoxicity.

4.3.6.7.1 Cytotoxicity in presence of chloramphenicol

Chloramphenicol is known to inhibit the translation of mRNA in bacteria as a result of binding to 50S ribosomal subunit during protein biosynthesis and blocks the peptide chain elongation. (Schlünzen *et al* 2001; Schwarz *et al,* 2004; Montero *et al,* 2007). This means that Chloramphenicol does not kill the bacteria immediumtely and bacteria might be able to continue activity for some time but are not able to produce any new proteins. This allows determination whether new proteins synthesis is required to initiate toxicity

Human H4 cells were incubated with the cytotoxic *C. sakazakii* ST3 strain 978 and selected bacterial isolates (*K1* strain 939, *C. condimenti* strain 1330, *C. sakazakii* ST3 strain 984 and *Salmonella* Enteritidis) in presence of 20 µg/ml Chloramphenicol for 3h at 37^{o}C in tissue culture incubator. Surprisingly the cytotoxicity was completely loss in same isolates, despite the minor reduction in bacterial cells number at the end of experiment was observed in the wells treated with Chloramphenicol (table 4-2). The data suggests that the bacteria have to be live and able to produce toxic agents to be able to destroy human cells.

Table 4-2 Effect of chloramphenicol on bacterial growth and cytotoxicity for H4 cells.

strain	Without Chloamphenicol			With Chloamphenicol		
	cytotoxicity%	log cycle at 0h	log cycle at 3h	cytotoxicity%	log cycle at 0h	log cycle at 3h
C. sakazakii ST3 984	2.4	7	9	2.4	6	6
C. sakazakii ST3 978	80.9	5	9	0.0	7	7
C. condimenti 1330	10.1	8	8	2.9	8	7
E.coli K1 939	72.2	7	8	0.0	8	7
E.coli K1 1230	3.1	7	7	0.0	7	6
S. Enteritidis 538	11.8	8	8	0.0	7	7

Bacterial strains were incubated with H4 cells in presence/absent of 20 µg/ml Chloramphenicol antibiotic in DMEM medium for 3h at 37^{o}C in tissue culture incubator to determine whether production of new proteins is required for this high level of cytotoxicity recorded for *C. sakazakii* ST3 strain 978. Bacterial density for each strain was determined by serial dilution in TSB at time 0 and after 3 hours to investigate the effect of Chloramphenicol on bacterial survival during the assay and represented as log cycle. Minor reduction in the bacterial number was observed with *C. condimenti* and *E. coli* K1, whereas the cytotoxicity was almost depleted

Table 4-3 Effect of chloramphenicol in bacterial growth and cytotoxicity for Caco-2 cells.

strain	Without Chloamphenicol			With Chloamphenicol		
	cytotoxicity%	log cycle at 0h	log cycle at 3h	cytotoxicity%	log cycle at 0h	log cycle at 3h
C. sakazakii ST3 984	2.3	7	9	1.7	6	6
C. sakazakii ST3 978	69	5	9	2.35	7	7
C. condimenti 1330	5	8	8	2.3	8	7
E.coli K1 939	66	7	8	2.1	8	7
E.coli K1 1230	1.6	7	7	0	7	6
S. Enteritidis 538	10	8	8	1.3	7	7

Bacterial strains were incubated with Caco-2 cells in presence/absent of 20 µg/ml Chloramphenicol antibiotic in DMEM medium for 3h at 37°C in tissue culture incubator to determine whether production of new proteins is required for this high level of cytotoxicity recorded for *C. sakazakii* ST3 strain 978. Bacterial density for each strain was determined by serial dilution in TSB at time 0 and after 3 hours to investigate the effect of Chloramphenicol on bacterial survival during the assay and represented as log cycle. Minor reduction in the bacterial number was observed with *C. condimenti* and *E. coli* K1, whereas the cytotoxicity was almost depleted.

4.3.6.7.2 Role of direct contact in bacterial cytotoxicity.

As it was determined that extracellular toxins were not responsible for killing the human cells, and the killing was not as a result of physical contact as live cells were not able to kill human cells in presence of proteins inhibitor chloramphenicol, the idea of this experiment was to investigate the effect of incubation of human cells and bacteria in same environment and prohibit bacteria from direct contact with human cells using 6.5 mm Transwell with 0.4 µm pore polycarbonate membrane insert (Sigma, UK), since the size of *Cronobacter* ranged 1 µm by 3 µm as described (Farmer *et al,* 1980; Iversen *et al* 2007).

Table 4-4 Role of direct contact in bacterial cytotoxicity.

Strain	Cytotoxicity% with bacteria in direct contact with cells		Cytotoxicity% of bacteria added to the upper chamber	
	H4 cells	Caco-2 cells	H4 cells	Caco-2 cells
C. sakazakii ST3 984	**3.3**	**2.4**	**2.4**	**3.5**
C. sakazakii ST3 978	**8 1.6**	**71.6**	**3.5**	**4.5**
C. condimenti 1330	**8.9**	**4.8**	**4.4**	**2.2**
E.coli K1 939	**74**	**70.4**	**4.6**	**5.2**
E.coli K1 1230	**2.9**	**2.7**	**2.7**	**2.2**
S. Enteritidis 538	**12.5**	**10.1**	**4.2**	**4**

Bacterial cytotoxicity was investigated either in direct contact when bacteria added directly to confluent monolayer of H4 and Caco-2 cells or with bacteria added to the upper chamber of 3 µm inserts of confluent monolayer of H4 cells and cytotoxicity compared. Results showed that direct contact is essential to initiate cytotoxicity.

Inserts were placed in a 24-well plates with 100% confluent H4 or Caco-2 cells. 200 µl of OD_{600} of 0.05 prepared from 18-24h broth of *C. sakazakii* ST3 984 and 978, *C. condiment* 1330, *E. coli* K1 939, *Salmonella* Enteritidis 538 and *E. coli* K12 strain 1230 bacterial suspension at the same concentration were carefully added to the upper chamber of the inserts. Fresh medium was added to negative control inserts (non-treated cells). After 3h incubation, human cell viability was calculated using Trypan blue dye. Interestingly, no cytotoxic activity was observed for the investigated strains and the results was close to non-treated cells (Table 4-4). This finding indicates that: bacterial cells have to be present, active and in contact with human cells, which might indicate to the roll of interaction between host and pathogen and the role of type secretion systems in this bacteria.

4.3.6.7.3 Genomic comparison of ST3 isolates.

To explain these interesting findings, a genome comparison using MLST database (https://pubmlst.org/*cronobacter*/) was applied between the two-sequenced ST3 isolate (978 and 984) which were both isolated from neonatal enteral feeding tube. Unfortunately, the results obtained from genome analysis were not enough to

discriminate between these two isolates using Artemis Comparison Tool webACT software, and both strains were subjected for new whole genome sequence.

Strains were grown on a TSA plate and DNA extracted and purified as described in section 2.8.4.12.1. Whole genome sequence was performed at NTU microbial genome research lab by research group. Genomic DNA libraries were prepared using the Illumina Nextera XT DNA Library Preparation Kit and Nextera XT v2 Index Kit set A. The genomes were sequenced on the Illumina MiSeq platform, using a MiSeq v2 Reagent Cartridge.

The number of annotated genes per genome varied between 4234 genes for strain 978 and 4227 for strain 984. After annotation of the obtained genomes, the seven housekeeping genes used in MLST were firstly extracted to confirm the sequence type and results confirmed that both strains were ST3. Then presence/absence of genes were compared using webACT program was conducted (Abbott, Aanensen and Bentley, 2007), and the results obtained as an excel sheet containing all genes with brief description. Results indicated that both strains shared about 99% of their genes and there were only 63 genes differences, some of them with were of unknown function or hypothetical proteins. Among these 63 genes, 37 were unique for the virulent strain 978 (table 4-5), while 26 genes were found in non-virulent strain 984 and not in strain 978 (Table 4-6). A multiple search in the website data base was conducted to find any possible genes linked to this virulence, but however no direct or clear link wasobtained. However, the genes with unknown function and stated as encoding for hypothetical proteins might be involved in this serious pathology.

Table 4-5. The unique genes of virulent C. sakazakii ST3 isolates 978.

no	Gene	FUNCTION
1	group_12	DNA polymerase V subunit UmuC
2	group_14	Phage-related baseplate assembly protein
3	group_16	hypothetical protein
4	lexA_3	LexA repressor
5	lexA_5	LexA repressor
6	dnaX_2	DNA polymerase III subunit tau
7	ybhN_2	Inner membrane protein YbhN
8	hcpA_4	Major exported protein
9	katG_2	Catalase-peroxidase
110	katG_2	Catalase-peroxidase
11	selU_2	tRNA 2-selenouridine synthase
12	group_3975	hypothetical protein
13	fabF_3	3-oxoacyl-[acyl-carrier-protein] synthase 2
14	group_4144	hypothetical protein
15	group_4164	hypothetical protein
16	group_72	hypothetical protein
17	group_73	Carbohydrate binding domain protein
18	lpxK_1	Tetraacyldisaccharide 4'-kinase
19	ada_1	Bifunctional transcriptional activator/DNA repair enzyme Ada
20	group_76	hypothetical protein
21	chiA_2	Chitinase A precursor
22	group_78	hypothetical protein
23	selA_3	L-seryl-tRNA(Sec) selenium transferase
24	group_76	hypothetical protein
25	chiA_2	Chitinase A precursor
26	group_78	hypothetical protein
27	selA_3	L-seryl-tRNA(Sec) selenium transferase
28	tuf1_1	Elongation factor Tu 1
29	group_80	hypothetical protein
30	group_81	hypothetical protein
31	group_82	hypothetical protein
32	group_83	hypothetical protein
33	gadB	Glutamate decarboxylase beta
34	group_85	Capsid protein (F protein)
35	group_86	hypothetical protein
36	group_87	hypothetical protein
37	metH_2	Methionine synthase

Table 4-6. The unique genes of non-virulent *C. sakazakii* ST3 isolates 984.

	Gene	FUNCTION
1	pksB	putative polyketide biosynthesis zinc-dependent hydrolase PksB
2	group_101	hypothetical protein
3	acsA	Acetyl-coenzyme A synthetase
4	group_11	DNA polymerase V subunit UmuC
5	group_17	hypothetical protein
6	group_21	hypothetical protein
7	ssa1	Serotype-specific antigen 1 precursor
8	dcp_1	Peptidyl-dipeptidase dcp
9	tuf1_1	Elongation factor Tu 1
110	tuf1_2	Elongation factor Tu 1
11	xerC_4	Tyrosine recombinase XerC
12	group_33	hypothetical protein
13	ada	Bifunctional transcriptional activator/DNA repair enzyme Ada
14	ada	Bifunctional transcriptional activator/DNA repair enzyme Ada
15	hcpA_4	Major exported protein
16	group_89	hypothetical protein
17	group_90	Phage antitermination protein Q
18	mobB_2	Molybdopterin-guanine dinucleotide biosynthesis adapter protein
19	group_92	IS2 repressor TnpA
20	group_93	hypothetical protein
21	lexA_4	LexA repressor
22	group_95	hypothetical protein
23	group_96	hypothetical protein
24	group_97	hypothetical protein
25	group_98	Integrase core domain protein
26	group_99	putative Permease Membrane Region

4.3.7 Role of eukaryotic cytoskeleton in bacterial invasion.

Several pathogenic bacteria can use host cell components and/or disrupt the cellular functions for invasion. Moreover, uptake by host cell might involve bacterial infection and facilitate its invasion. Rearrangement of actin cytoskeleton in host cell is one of the common strategies that can be used by different enteropathogenic bacteria such as *Salmonella, Listeria, Shigella* and *Yersinia* for internalization process (Navarro-Garcia *et al,* 2013; de Souza Santos and Orth, 2015 and Mittal *et al,* 2016). Cytoskeletal actin is responsible for motility and shape in eukaryotic cells and many other essential cellular processes (Etienne-Manneville, 2004).

However, actin polymerization can be induced by some pathogenic bacteria such as *Listeria* and *Shigella* to propel the invaded bacteria through the cytoplasm into nearby cells (Cossart, 2000; 2001; Cossart and Sansonetti, 2004; Guiney and Lesnick, 2005; Ferreira *et al,* 2006 and Mittal *et al,* 2016)). Therefore, the role of host cells in this process was investigated, and the effect of the human cytoskeleton inhibitors on the invasion mechanism and how they affect the number of internalized bacteria was investigated.

Three different eukaryotic cytoskeleton inhibitors were used in this assay which are: Cytochalasin D (2 µM) which is a Microfilaments depolymerisation agent, Nocodazole (20 µM) and Colchicine (10 µM) which all are eukaryotic microtubules inhibitors. These concentrations were optimized in separate experiments and incubated with the cell lines for the appropriate periods according to Mohan Nair and Venkitanarayanan, (2007).

Twelve Cronobacter strains including the seven-type species with NTU collection and another five isolates were chosen according to their clinical history or according to their level of invasion in previous assays. In addition, *E. coli* K1 NTU strain 939 used as a comparative strain from different genus, and because this particular serotype is known to be responsible for neonatal meningitis. *Salmonella* Enteritidis was used as a positive control of invasion assay.

4.3.7.1 Role of eukaryotic cytoskeleton in *Cronobacter* type species invasion to H4 cell line.

The effect of human cytoskeleton on *Cronobacter* seven type species was varied, and only microtubules inhibitor colchicine was observed to inhibit all of the investigated isolates but to variable levels.

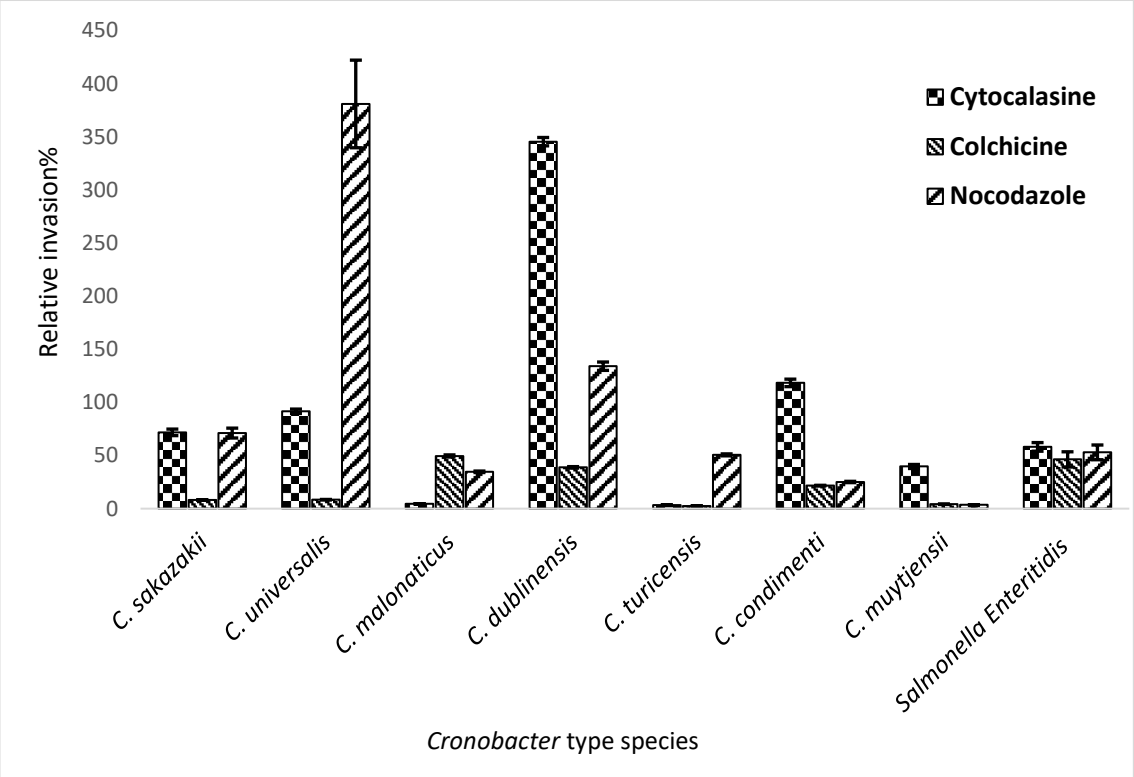


Figure 4-20 Effect of cytoskeleton inhibitors on the invasion of *Cronobacter* 7 species into H4 cells. Human cells were pre-incubated with stated inhibitors [Cytochalasin D (2 µM), Nocodazole (20 µM) and Colchicine (10 µM)] as described by Mohan Nair and Venkitanarayanan, (2007). And then infected with bacteria for 3h. The data presented in mean±standard error of mean of two independent experiments. Data presented as % invasion of 100% control of each strain where no inhibitors were added to untreated cells.

Microfilaments inhibitor Cytochalasin D inhibited the invasion of most of *Cronobacter* type species isolate except *C. dublinensis* and *C. condimenti* which showed about 345% and 118% increase in the number of invaded bacteria compared with control respectively. Invasion of *C. turicensis and C. malonaticus* was highly inhibited by Cytochalasin D and 97% and 95% reduction in invasion compared to untreated cells respectively was observed, about 60% of *C. muytjensii* invasion was inhibited by Cytochalasin D, while only 9% inhibition was recorded by *C. universalis* and 29% for *C. sakazakii.*

The number of internalized bacteria was largely inhibited by the microtubules inhibitor colchicine from about 50% for *C. malonaticus* and up to 97.5% inhibition (only 2.5% of invaded bacteria compared to non-treated cells) shown by *C. turicensis. C. sakazakii, C, universalis* and *C. muytjensii* were all inhibited by more than 90% compared with untreated controls.

Additionally, the effect of microtubules formation inhibitor Nocodazole was also variable. This inhibitor caused an increase of the invasion level of some strains like *C. universalis* (380%) and *C. dublinensis* (138%), whereas other type species were variably inhibited (Figure 4-19). *C. muytjensii* was the most inhibited strain, as only 5% of bacteria were able to invade compared with the untreated cells, while inhibition of 29% was observed by *C. sakazakii*. *C. malonaticus, C. turicensis* and *C. condimenti* revealed 65%, 50% and 75% inhibition respectively. In general, the invasion of the seven *Cronobacter* type species was clearly effected with human cytoskeleton inhibitors and according to the results some strains showed partial dependence of their invasion on the host cell components, while other strains were most likely suppressed by loss of actin cytoskeleton function.

4.3.7.2 Role of eukaryotic cytoskeleton in *Cronobacter* type species invasion to Caco-2 cell line.

With respect to Caco-2 cells, the investigated strains displayed a different manner of their invasion to treated cells compared with results obtained by H4 cells. For example: only two strains were inhibited by Cytochalasin D which are *C. turicensis* and *C. condimenti* with about 50% and 35% reduction respectively, while invasion of other strains was enhanced compared to untreated control. The invasion of *C. sakazakii malonaticus, C. universalis C. dublinensis* and *C. muytjensii* was increased for about 110% compared to untreated control (Figure 4-21).

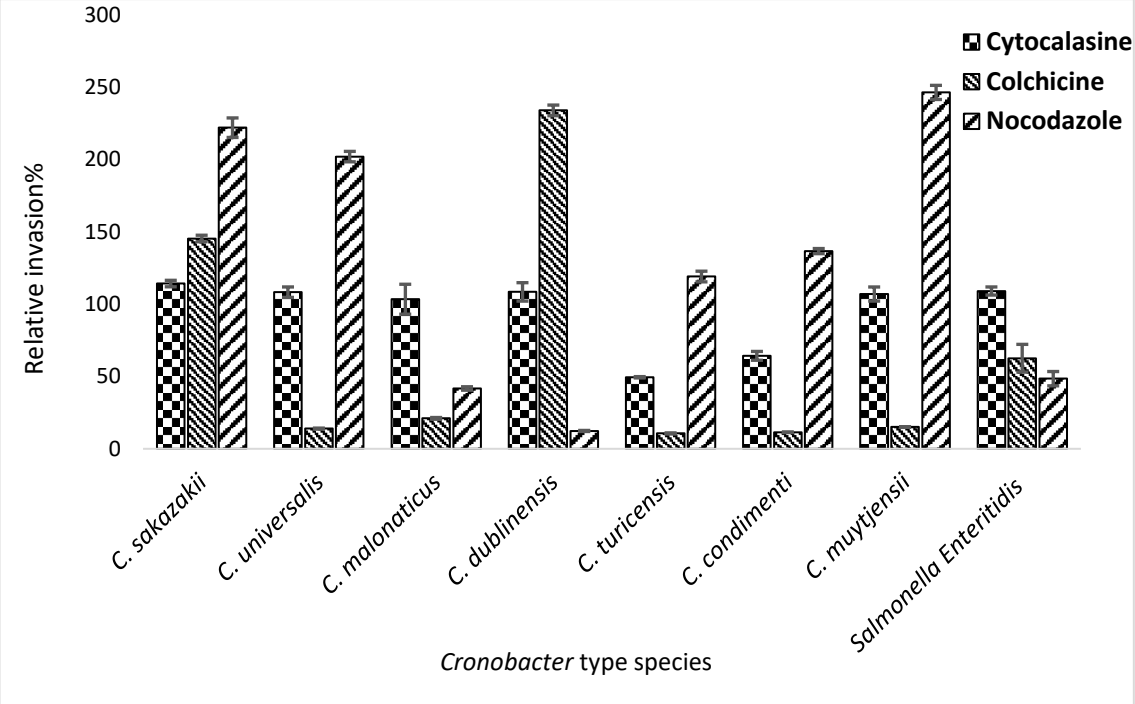


Figure 4-21 Effect of cytoskeleton inhibitors on the invasion of *Cronobacter* 7 species to Caco-2 cells. Human cells were pre-incubated with stated inhibitors [Cytochalasin D (2 µM), Nocodazole (20 µM) and Colchicine (10 µM)] as described by Mohan Nair and Venkitanarayanan, (2007). And then infected with bacteria for 3h. The data presented in mean±standard error of mean of two independent experiments. Data presented as a% invasion of 100% control of each strain where no inhibitors were added to untreated cells.

Colchicine exhibited variable effects on the invasion of *Cronobacter* into Caco-2 cells which are different of that obtained by H4 cells. The invasion of *C. sakazakii* and *C. dublinensis* was increased for about 145% and 234% respectively, while the invasion of the rest of the five type species was suppressed to about 10% to 20%. Majority of *Cronobacter* type species (5/7) were enhanced by pre-incubation of human cells with Nocodazole inhibitor in regard to their invasion, and showed increase of up to250% compared to untreated control(shown by *C. muytjensii*). *C. sakazakii* and *C. universalis* were increased by 220% and 200% whereas *C. turicensis* and *C. condimenti* increased by 120% and 136% respectively. in contrast, the invasion of *C. malonaticus* was reduced to 40% invasion compared to untreated control and *C. dublinensis* displayed only 12% compared to untreated control.

4.3.7.3 Role of eukaryotic cytoskeleton in the invasion of selected pathogenic bacterial isolates to H4 cell line.

Results indicated that most of cytoskeleton depolymerisation agents were able to inhibit bacterial invasion to this cell line with exception of Nocodazole.

The microfilaments depolymerisation agent Cytochalasin D has inhibited the number of invasion bacteria with levels ranging from about 42% to for *Salmonella* Enteritidis to about 85% for *C. malonaticus*. Inhibition of other *Cronobacter* isolates ranged from 61% to 79%, while K1 strain 939 inhibited by 42% from the untreated H4 cells (Figure 4-22).

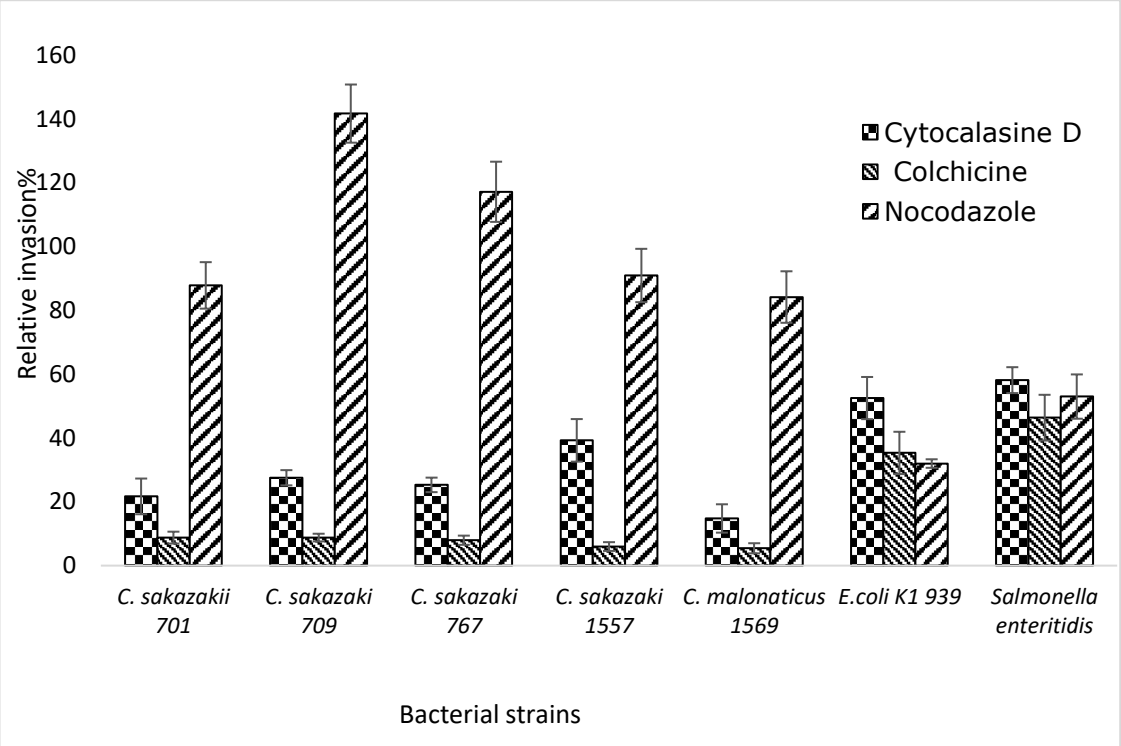


Figure 4-22. Effect of cytoskeleton inhibitors on the Invasion of selected bacterial isolates to H4 cells. Human cells were pre-incubated with stated inhibitors [Cytochalasin D (2 µM), Nocodazole (20 µM) and Colchicine (10 µM)] as described by Mohan Nair and Venkitanarayanan, (2007). And then infected with bacteria for 3h. The data presented in mean±standard error of mean of two independent experiments. Data presented as a% invasion of 100% control of each strain where no inhibitors were added to untreated cells.

Interestingly, the effect of Colchicine was nearly similar with all of *Cronobacter* type species isolates, and the average inhibition was from 91% to 94%, while *Salmonella* Enteritidis strain 358 and K1 strain 939 were inhibited by almost 53% and 54% respectively. However, the inhibition of bacterial invasion by microtubules inhibitor Nocodazole was completely different, and the number of invasion bacteria to H4 cells was increased up to 141.6% and 117.1% for *C. sakazakii* strains 709 and 767 respectively.

These two strains were able to enter to the bloodstream and develop septicaemia and meningitis respectively. Nocodazole has inhibited the invasion of *E. coli* K1 strain 939 and *Salmonella* Enteritidis strain 358 to about 69% and 48% of the untreated cells respectively (Figure 4-22).

4.3.7.4 Role of eukaryotic cytoskeleton in the invasion of selected pathogenic bacterial isolates to Caco-2 cell line.

The effect of cytoskeleton inhibitors on the bacterial invasion to Caco-2 cell was completely different to the results obtained by H4 cell line as shown in Figure 4-23.

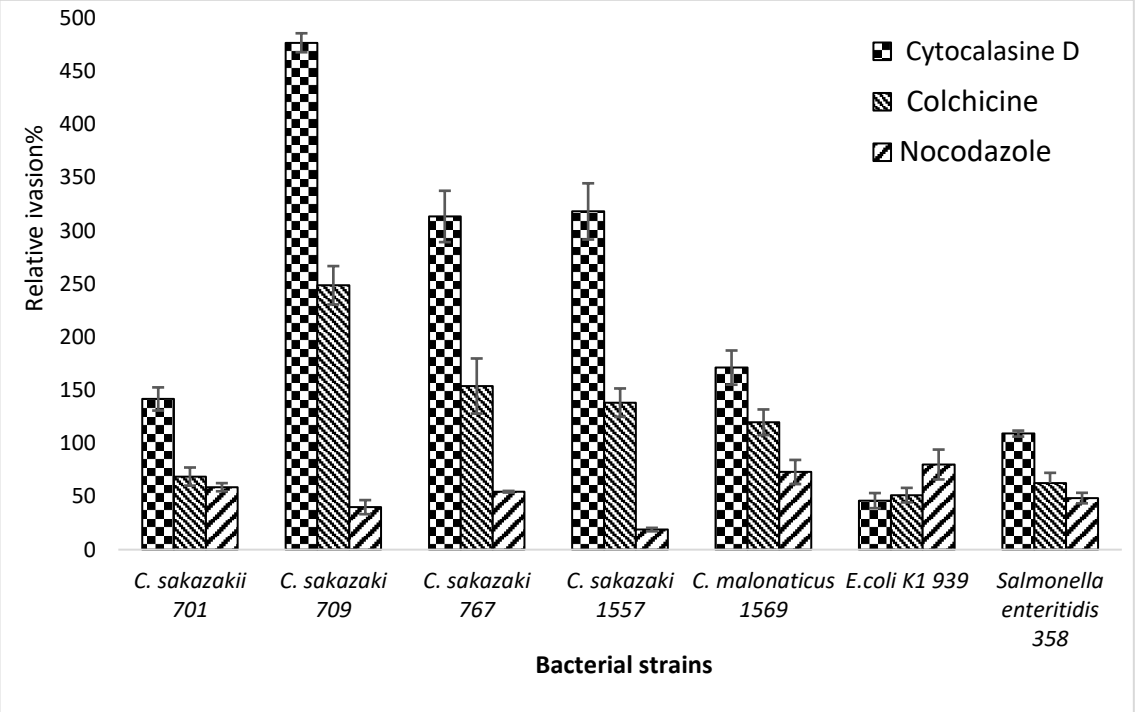


Figure 4-23. Effect of cytoskeleton inhibitors on the Invasion of selected bacterial isoaltes to Caco-2 cells. Human cells were pre-incubated with stated inhibitors inhibitors [Cytochalasin D (2 µM), Nocodazole (20 µM) and Colchicine (10 µM)] as described by Mohan Nair and Venkitanarayanan, (2007). And then infected with bacteria for 3h. The data presented in mean±standard error of mean of two independent experiments. Data presented as a% invasion of 100% control of each strain where no inhibitors were added to untreated cells.

Cytochalasin D enhanced the number of internalised bacteria to Caco-2 fivefold higher than the controls for *Cronobacter sakazakii* strain 709 (476%) and about 3 times fold for *C. sakazakii* strains 767 and 1557 (313% and 318% respectively). *C. sakazakii* strains 701 and *C. malonaticus* strain 1569 showed an increase of the number of entered bacteria of approximately 141% and 171% respectively. While the increase of invaded *S.* Enteritidis was minimal, K1 strain 939 showed a different result and the number of

internalised bacteria reduced to 46% compared with no Cytochalasin D added (Figure 4-23).

Moreover, when the MT depolymerisation agent colchicine was used, the invasiveness of *C. sakazakii* strains 709, 767, 1557 and *C. malonaticus* strain 1569 was also increased by up to 250%, 153%, 138% and 119% respectively. Interestingly, *C. sakazakii* ST4 strain 701, although sharing the same sequence type with strains 709 and 767 it was inhibited by 42% when compared with invasion in absence of any inhibitor.

Nocodazole, the last inhibitor in this assay also showed different effects from that obtained from H4 cell line. While it enhanced the invasion of some *Cronobacter* strains to H4 cell line, invasion Of Caco-2 cells was decreased in all *Cronobacter* strains as well as and *S. enteritidis.* For example, *C. sakazakii* strains 1557 and were inhibited by 81% and 60% respectively. Other *Cronobacter* strains showed inhibition levels ranged from 28% to 46% while 939 showed less inhibition by this chemical which is only 20% and about 50% for the positive control strain 358 (Figure 4-23).

4.4 Discussion.

Human cell lines are often used in investigating host-pathogen interaction. Different cell lines have been established for this purpose, but however many of them were derived from cancer cells. Caco-2 cells has been used worldwide in an extensive range of bacterial infections disease experiments, for example, Almajed and Forsythe, (2016) reported the overcoming by *C. sakazakii* clinical isolates of host barriers and evasion of immune response using different cell lines including Caco-2. However, Caco-2 cells are an immortalised cell line and are derived from an adult colon carcinoma (Buhrke *et al,* 2011), which may not match and mimic the reality of pathogenesis mechanisms in healthy and immature cells like that in neonates (Nanthakumar *et al,* 2000; Claud *et al,* 2003; Savidge *et al,* 2006). In addition to Caco-2 cell line, HT-29, T84, and HCA7 intestinal epithelia cell lines were derived from human colon carcinoma and widely used in the host pathogen interaction (Bambou *et al,* 2004: He *et al,* 2007).

H4 cell line was established as new model of non-malignant neonatal epithelia intestinal cell line by Sanderson and Walker (1995). Later, Sanderson *et al,* (1996) reported that

this cell line expressed villin and cytokeratins as proof of their epithelial cell source. Different studies have compared H4 cell with adult in regard to the inflammatory response such as Caco-2 and HT29-cl19A and concluded that H4 produces cytokines significantly higher than adult cell lines (Nanthakumar *et al,* 2000; Claud *et al,* 2003; Savidge *et al,* 2006).

4.4.1 Optimization of Tissue culture assay

Cell lines were firstly investigated for their required time to reach at least 80% confluent on 24-well plates according to each recommended protocol, and results revealed that H4 cells were able to reach 100% confluence between 18-24h, while Caco-2 needed about 48hrs to cover up to 90% of the 24-well plates (see Figure 4-1). However, this is an essential step for experimental design as it is very important to determine the confluent time in order to add the bacteria to avoid developing of multiple layers by the chosen cell line, and also to avoid using non-confluent cells that could result in increased attach and invaded results (Mooney *et al,* 2003).

Due to the limited data available about the H4 cell line with regard to the effect of passage number on cell line characteristics, the invasion of selected bacterial strains to high passage (48-51) and low passage (17-19) was investigated.

Although *C. sakazakii* strain 703 (NECII) and 767 (meningitic) strains were slightly more invasive to low passage cells, no significant differences using paired t test (P= 0.3008), or specific pattern of invasion to low or high passage numbers as shown by other strains. This indicate that the serial passage does not affect the cells phenotype in this regard, and may not affect bacterial interaction with this cell line. This however, is in contrast with results obtained by Lee and Falkow (1990) when the adherence and invasion of *Salmonella* isolates were 10-fold lower when low passages (8-10) of Madin-Darby Canine Kidney (MDCK) Cells were used compared with high passage (40-60).

In addition, O'Driscoll *et al,* (2006) compared the differences in mRNAs expression of low passage (passage 18) and high passage (passage 40) of Pancreatic beta cell line (MIN-6) and found significant differences in the expression of about 1,000 genes between low and high passage cells. Moreover, differences were observed in the

expression of green fluorescent plasmid (GFP) between low and high passages of Caco-2, as high passages showed an increase of the expression while, MCF7 cell line displayed decreased expression of GFP as passages increased (Atcc.org, 2017).

The similarity of bacterial invasion to high or low H4 passages might be because the H4 cell line is non-malignant, which might has better efficiency and stability even at high passages compared with transformed and malignant derived cell lines, that might change over the serial passages. However, passages used in this project were from 6 to 20, either for H4 cell or Caco-2 cells.

4.4.1.1 Attachment assay.

The selected bacterial strains were examined for their attachment to H4 and Caco-2 cells cell lines. Most strains were more adherent to H4 cells than Caco-2 cells ($P<0.05$), and most strains showed significant attachment compared to negative control *E. coli* K12. Three strains with serious clinical symptoms were among the top 10 adhesive isolates to H4 cells which are *C. sakazakii* strains 696 and 703 well as the *C. malonaticus* 1569. In contrast, only two strains associated with serious pathology were among the top 10 adhesives to Caco-2 (*C. sakazakii* strains 696 and *C. malonaticus* 1569). Both *C. sakazakii* ST12 strains 703 and 696 associated with neonatal NECII were among the highest adhesive strains to H4 cells among *C. sakazakii* strains and revealed 52% and 36% compared with only 5%and 17.6% adhesion to Caco-2 cell respectively.

Forsythe *et al,* (2014) demonstrated that ST12 has been associated with cases of necrotizing enterocolitis in neonates, and the high adhesion observed by ST12 isolates is a potential factor of exaggerated host response and increase the possibility of NEC incidence.

Most of *C. malonaticus* isolates were more adhesive to H4 cell than *C. sakazakii* ST4 and ST8 strains. *C. malonaticus* strain 1569 ST307 presented about 42%, while *C. malonaticus* ST7 strains 681 and 1558 presented 28% and 25% respectively. However, *C. malonaticus* 1569 was the first reported isolate from neonatal meningitis, while *C. malonaticus* ST7 is more likely associated with adult and infection in the immunocompromised (Forsythe *et al,* 2014; Holy *et al,* 2014; Alsonosi *et al,* 2015).

In general, *C. sakazakii* and *C. malonaticus* strains were the highest adhesive to H4 cells among *Cronobacter* isolates, which is broadly in agreement with previous suggestion of the association of these two species with clinical pathologies (Holy and Forsythe, 2014; Jackson *et al,* 2014). In summary, *E. coli* K1 strain 939 was the highest adhesive strain to H4 cells and displayed about 94% of the inoculum.

C. sakazakii strains 696, 701, and 563, and *C. malonaticus* strain 681 as well as *E. coli* K1 939 were among the top adhesive strains and displayed high growth rate, whereas some strains that displayed high adhesion level showed slower growth including. *C. sakazakii* 703, 978 *C. malonaticus* 1569 and *C. universalis* 581, suggesting no clear link between bacterial attachment and its growth rate.

Capsule production is a potential virulence factor and thought to contribute in the ability of bacteria to cause disease including attachment to host cells and other environmental surfaces by different bacterial species including *Cronobacter* spp. (Hurrell *et al,* 2009; Ye *et al,* 2016). Most of strains that displayed moderate to high capsules, especially on the milk agar medium were further moderate to high adhesive to both cell lines. This was noted by clinically significant *Cronobacter* sakazakii strains including 767 (meningitis), 709 (septicaemia), and 701 and 703 (NEC), as well as *C. malonaticus* strain 1569 (meningitis).

Yet, this is not constantly true, as some strains with unknown pathology or those showed non- significant clinical signs was also among the top adhesive and capsule producing strains such as *C. sakazakii* strain 563 and *C. malonaticus* 681. However, some clinically significant strains such as *C. sakazakii* 1249 (meningitis), and *C. turicensis* 1211(meningitis) displayed low attachment to both cell lines, low to moderate growth rate, yet were moderate to high capsule producers.

Although, previous studies indicated a role of flagellum and fimbriae in bacterial adhesion to host cells (Hartmann *et al,* 2010), no correlation was observed between the level of bacterial attachment and bacterial motility in the present study, as most of strains that showed moderate to high motility revealed low level of attachment, and in contrast *C. universalis* strain 581 which is non-motile was comparatively high adhesive to H4 cells. In addition, no link was observed between the presences of curli fimbriae

detected by Congo red assay and level of attachment, and for example *C. sakazakii* 716 was positive in curli fimbriae and among the lowest adhesive strains, whereas strain 696 did not express curli fimbriae and was among the most adherent isolates especially to H4 cells.

In general, most strains displayed attachment to Caco-2 cells less than 50% of that recorded with H4 cell line (Figure 4-4). For example, *C. sakazakii* ST4 strains 701 showed adhesion to Caco-2 fivefold less than H4 cells, while *C. sakazakii* ST41 strain 563 displayed adhesion to Caco-2 eight fold less than that shown with H4 cells.

As mentioned previously, *C. sakazakii* ST12 strain 696 *C. malonaticus* 1569 were among the highest adhesive strain to Caco-2 cells among *Cronobacter* isolates, which are NEC and meningitic isolates respectively. *C. sakazakii* strains 701 (ST4/fatal NEC III) was number 12th in the adhesion to H4, and 22nd with Caco-2 cells. This difference needed to be taken in consideration, as might the response of neonates to pathogens is different of that displayed by adults, and as clinical strains were more significantly adhesive to H4 cell, it could be possible that this cell line is better model in this field of research, especially neonatal infections investigations.

In comparison with *E. coli* K1 strain 939, although it was the highest adhesive strain to Caco-2 cells, the adhesion was less than 50% of that recorded with H4 cell line.

Townsend *et al.* (2008) examined the attachment and invasion of different *C. sakazakii* (*Enterobacter sakazakii*) including *C. sakazakii* ST12 strain 696 and ST4 strains (701, 709 and 767) to Caco-2 cell line and showed 2.6% and average of (0.140–.96%) respectively. Which is lower than the level showed in the present study. This might because of the experimental condition and/or the unknown passage number of cellsused in their study.

4.4.1.2 Invasion assay.

All *Cronobacter* strains were able to invade H4 cell line except *C. condimenti* strain 1330 (type species isolate) which can be considered as a non-invasive strain. Cruz-Cordova *et al,* (2012) indicated that to date *C. condementi* was not associated with any infection.

In general, when strains are arranged according to the level of invasion to both cell lines, five clinically significant strains were among the top 10 invasive to H4 cell line. Three of these strains were from French outbreak isolates, which are 701, 709 and 767, and associated with pathologies NECIII, septicaemia and meningitis respectively. The other two isolates were meningitic isolates and are *C. malonaticus* strain 1569 and *C. turicensis* strain 564. These strains were motile, biofilm producing and highly capsulated in different agar medium especially milk agar. Among these strains, *C. sakazakii* strain 701, *C. malonaticus* 1569, and *C. turicensis* 564 were curli fimbria positive according to Congo red assay and displayed RDAR phenotype at 37^{o}C (Table 3-2).

C. sakazakii strain 1557 was the highest invasive strain to H4 cells, this strain was isolated from bronchial secretion of child with respiratory system infection, and according to the high invasion level reported in the present study, this strain could case very serious illnesses, and if allowed to reach to blood stream it might overcome host brain barrier and cause meningitis. In addition, the meningitic isolate *C. turicensis* ST5 strain 564 was most invasive strain to H4 cells among the non *C. sakazakii* isolates, giving strong association between the level of invasion and this disease.

Low invasion was observed by isolates with weak or unknown clinical history such as *C. sakazakii* ST8 strains 1 and 513, which is been also reported by Almajed and Forsythe (2015). Although, this serotype is normally isolated from clinical sites, it is not associated with significant infections (Hariri *et al,* 2013; Almajed and Forsythe, 2015).

Generally, strains of *C. sakazakii*, *C. malonaticus* and *C. turicensis* were the most invasive strains into H4 cells with significant differences ($P<0.0001$), while there was no significant differences between the other *Cronobacter* species such as *C. universalis, C. condimenti* and *C. dublinensis*. Moreover*, C. sakazakii* ST4 isolates showed high level of invasion to both cell liens compared with that with non-ST4 isolates, whereas ST12 showed more adhesion to both cell lines. ST4 strains were predominantly associated with neonatal meningitis, while ST12 isolates linked to NEC, which is in agreement with the resent study findings. No clear correlation was observed between adhesion and invasion and as an example, strain 1557 is one of the least adhesive, yet the most invasive strains to H4 cell line. H4 invasion results were higher than the invasion to Caco-

2 reported by Townsend *et al,* (2008) when strains 701 a NEC III isolate and 767 a meningitic isolate invaded at values, 0.17% and 0.23% of the inoculum compared with 0.8% and 0.5% into H4 respectively.

Strains were also investigated in regard to their ability to invade Caco-2 cells and results were varied. Interestingly, strains that were among top 10 invasive to H4 cell was also among the top 10 invasive to Caco-2 except with *C. turicensis* strain 1211 instead of 564. These two strains are from same species and isolated from fatal infant infection and neonatal meningitis respectively. Most of clinical strains showed high similarity in their pattern of invasion with slight changes in their position invasion level between cell lines. In general, some strains were more invasive to H4 and vice versa. The invasion of *Cronobacter* ST4 767 and 709 to Caco-2 cells were slightly higher than H4 cells, whereas the invasion of strain 701 (NEC III) were approximately similar in both cell lines. Most of *C. malonaticus* isolates (75%) were more invasive to Caco-2 cells than H4, and *C. turicensis* strain 1211 which is fatal infant infection isolate, was the most invasive to Caco-2.

Invasion results could be affected by cytotoxicity as some strains were recorded high levels of cytotoxicity confirmed by uptake of Trypan blue, which is mean that human cell membrane was damaged and gentamicin could possibly enter and kill the intracellular bacteria. Cytotoxicity will have discussed later in this chapter. Differences in bacterial adhesion and invasion stated in Figur4 4-5.

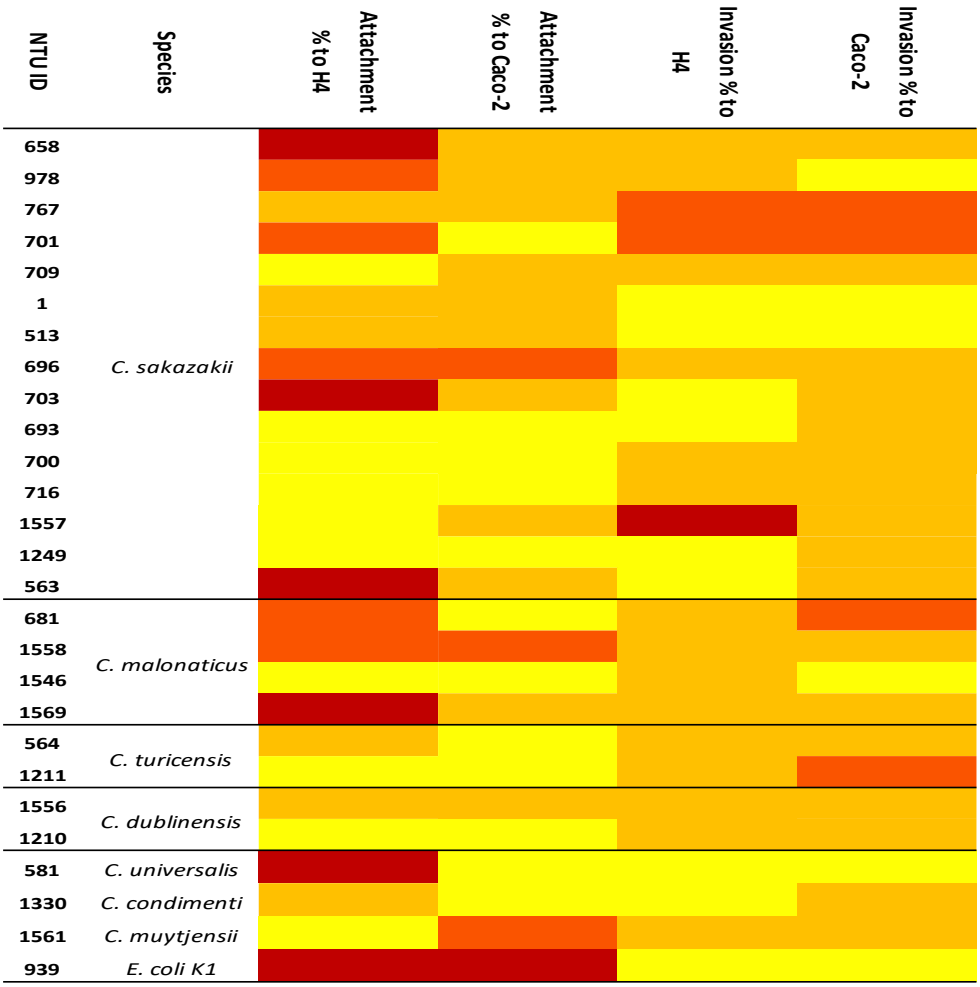


Figure 4-24. Summary of bacterial attachment and invasion results to H4 and Caco-2 cells. Results are indicated as heat map according to the strength of adhesion and invasion as shown below.

Attachment	invasion
low attachment <5%	low invasion <0.05%
moderate attachment <15	moderate invasion <0.1%
High attachment <30%	% High invasion <0.5
Very high attachment >30%	Very high invasion >1%

4.4.1.3 Bacterial translocation.

All of the investigated strains showed detectable levels of translocation through both cell lines except the negative control. However, the translocation of blood and meningitic isolates from different species was considerably higher through both H4 and caco-2 cells. These strains are *C. turicensis* 564 (meningitis), *C. malonaticus* 1569 (meningitis) *C. sakazakii* 767 (meningitis), *C. sakazakii* 1249 (meningitis), *C. turicensis* 1211 (fatal infant infection) *C. sakazakii* 701 (fatal NEC) *C. sakazakii* 709 (septicaemia),

as well as *E. coli K1* 939 (meningitic serotype). These strains were among the top 10 translocated isolates in both cell lines.

After three hours incubation with H4 cells *C. turicensis* 564 showed the highest translocation about 4.7%, followed by *C. malonaticus* 1569 (4%), and *C. sakazakii* strains 767, 1249, 701 and 709 showed 1.8 1.5%, 0.055% and 0.04% respectively, while *E. coli* K1 presented 2.3% of the inoculum. Some strains displayed more translocation through Caco-2 than H4 cells such as *C. sakazakii* 1249 and *E. coli* K1 isolate, while the translocation of strains 767 was approximately similar in both cell lines (Figure 4-10). However, despite that bacterial strains were significantly translocated through H4 cells higher than that showed with caco-2 cells after 1h incubation ($P<0.05$), no significant differences was obtained at 3h between the two cell lines.

C. sakazakii ST4 strain 767 was the third top highest translocated strains through polarized H4 cells. Translocation of the meningitic *C. sakazakii* ST4 strain 767 through both cell lines was considerably higher than other ST4 strains. This strain showed about 85 and 600 times higher translocation than the *C. sakazakii* strain 701 over H4 and Caco-2 respectively. This particular serotype was associated with neonatal meningitis over period of 30 years (Joseph & Forsythe. 2012; Hariri *et al,* 2013). Likewise, the translocation of meningitic *C. malonaticus* strain 1569 through H4 cells was higher than *C. sakazakii* ST4 strains 701 and 709 about 71 and 100 times respectively, while the translocation of this strain through Caco-2 cells was and about 526 and 255 times higher the than the *C. sakazakii* ST4 strains 701 and 709 through same cell line. This strain was the first and only the reported meningitis case due to *C. malonaticus* infection (Hariri *et al,* 2013), as well as showing the highest invasion of Caco-2 among *C. malonaticus* isolates suggesting that the traits used by this strain could be different from the other species members tested.

Although *C. sakazakii* ST 23 strain 1557 was the highest invasive strain to H4 cells, translocated about forty times less than the meningitic *C. sakazakii* strain 767. All strains showed more infiltration through H4 cell than Caco-2 cells after 1hincubation, and for example the translocation of *C. sakazakii* ST4 strain 767 through H4 cells was about 6

times higher than Caco-2 cells, *C. turicensis* strain 564 showed approximately 30 times, whereas *E. coli* K1 displayed about 70 times.

In general, strains can be divided into two groups according to their effect on the monolayers electric resistance TEER. First group includes strains translocated with major damage to the polarized monolayer including *C. sakazakii* strains 701, 709, 767 and 1249, and *E. coli* K1. However, this suggests that the increased translocation of these strains might occurs more easily through the pores on the damaged polarized cells, which may does not reflect the real translocation. The second group includes strains that showed high translocation which did not cause major damage to the cells, which are *C. sakazakii* 1557, *C malonaticus* 1569 and *C. turicensis* strain 564 and 1211. These strains might have applied the uptake strategy, or translocated extracellularly between the epithelial cells without effecting the cells viability or disrupting the tight junctions similar to that observed by *Klebsiella pneumoniae* (Hsu *et al,* 2014)

Regarding to the effect of these strains on Caco-2 polarization. Most strains did not cause major reduction in the TEER at the end of the experiment, except *C. sakazakii* 1249 that showed the highest translocation among *Cronobacter* isolates and *E. coli* K1, the most invasive strain. However, it was observed that H4 cells were more delicate than Caco-2 cells during the washing steps which could be an extra factor of the Caco-2 monolyers stability.

To conclude results of this assay, all strains used for translocation were clinically significant except *C. sakazakii* strain 693 was isolated from asymptomatic neonate and strain 1557 isolated from bronchial secretion and was the highest invasive strain to H4 cells. Three Meningitic isolates from three different species were among the top 5 translocated strains over both cell lines which are *C. sakazakii* 767, *C. malonaticus* 1569 and *C. turicensis* 564, suggesting the role of bacterial translocation over host barriers in meningitis occurrence, despite the differences on their effect on the monolayers polarization, which possible indicate to different mechanisms been used by theses strains to cross epithelial cells and translocate to deeper tissues.

4.4.1.4 Serum resistance:

After pathogenic bacteria successfully reach the bloodstream, they must be able to resist the host defenses mechanisms including phagocytosis, antimicrobial peptides and the complement system such as bactericidal activity of serum complement (Miajlovic *et al,* 2014). Serum lysozyme was believed to promote the lysis of encapsulated bacterial cells in presence of serum complement by the attacking of the bacterial cell wall (Callewaert and Michiels 2010). Serum killing assay was performed in 50% serum (v/v) to investigate the ability of strains used in this project to resist human serum complement components which is likely to be important after they have successfully invaded and translocated human epithelial cell line. Overall, most of strains displayed a sharp drop in the bacterial number (CFU) compared with initial numbers after exposure to serum.

The significant finding of this assay is that 12 strains most resist to serum were all *C. sakazakii* except one isolate *E. coli* K1 that was in the ninth most resistance. Three of *C. sakazakii* were associated with serious illnesses which are 1249 (meningitis), 709 (septicaemia) and 701 (NECIII), whereas the meningitic *C. sakazakii* 767 and *C. malonaticus* 1569 were among the 10 most susceptible to killing by serum bactericidal activity. Thus, these strains might have different strategies to escape serum effects and cause meningitis. *C. sakazakii* isolates were more resistant to serum than other *Cronobacter* isolates, and the highest survival was displayed by the meningitic *C. sakazakii* ST31 strain 1249, followed by strain 709 ST4 isolate from neonate with severe septicaemia, which were both amongst the highest capsule and biofilms producers, especially on milk based medium.

C. sakazakii ST12 strain 696 and ST14 strain 716 were completely sensitive and no bacteria were recovered on agar plate at all-time points. Interestingly these two strains were non-capsulated on XLD medium. Strain 696 produced very low capsule on milk-based medium compared with high capsulated strain 703 from same group of ST12 and was resistant to serum killing suggesting the role of capsule in serum resistance bacteria. This results in agreement with the previous studies that indicated the role of capsule in

bacterial serum resistance (Whitfield 2006; Buckles *et al,* 2009; Callewaert and Michiels 2010; Miajlovic *et al,* 2014).

BLAST search indicated that for most *C. sakazakii* strains, only *C. universalis* strain 581 possess the *Cpa* gene and showed a notable increase in bacterial survival at the end of serum resistant experiment. However, there was no clear correlation between carriage of this genes and experimental results, as the two *C. sakazakii* strains 1, 513 which both showed an increase in the bacterial numbers in human serum not have this gene, while strains 696 and 716 were positive for this gene and were sensitive to serum killing. Moreover, none of *C. malonaticus* possesses this gene and showed moderate resistance with exception of the type species ST7 strain 681, which was sensitive.

Generally, this suggests that different sequence types among same species can display different virulence. Moreover, Schwizer *et al,* (2013) indicated to the potential role of the hypothetical protein ESA_04103 in serum tolerance of *C. sakazakii,* and mutation in this gene resulted in significant reduction in this trait. Strains were searched for this gene and only *C. sakazakii* isolates were positive for this gene, which is broadly in agreement with Schwizer *et al,* (2013) as *C. sakazakii* isolates showed the highest level of serum resistance. However, this gene might not expressed in the two sensitive *C. sakazakii* strains, and homologous may be present in other species. This suggest no single gene is responsible for serum resistance and that virulence is not wholly based on the resistance to serum.

4.4.1.5 Cytotoxicity Result.

Cytotoxicity of the selected *Cronobacter* strains and *E. coli* K1 to H4 and Caco-2 cell lines was investigated using Trypan blue cell viability assay and Lactate dehydrogenase assay.

4.4.1.5.1 Trypan blue assay.

Cell viability results indicated that most of the investigated strains displayed a detectable level of cytotoxicity compared with the blank. Strains were categorized into three groups: non-cytotoxic those showed less than 1.5-fold increase in the cytotoxicity compared to the blank, moderate that showed between 1.5 and 4-fold increase in the cytotoxicity

compared to the blank, while the high cytotoxic are the strains that displayed more than 4-fold increase in the cytotoxicity compared to the blank . 16% of *Cronobacter* isolates were non-cytotoxic despite the high growth rate and adhesive such as the fatal NECIII *C. sakazakii* strain 701, suggests that this strain does not kill host cells, and instead of that exploit host mechanisms to induce long term inflammatory response that led to immune diseases including NECIII.

Unexpectedly, *C. sakazakii* ST8 strain 1 displayed moderate cytotoxicity to both cell lines, although it was non-haemolytic (section 3.3.7). Only Three *Cronobacter* isolates and *E. coli* K1 were high cytotoxic to H4 cells including *C. condimenti* 1330 (6-fold) and *C. sakazakii* 716 (4-fold). However, the low invasion shown by these strains might be because of their toxicity and subsequently the invaded bacteria were killed during the gentamicin protection assay. There also potential link between the cytotoxicity and disruption of polarised monolayers TEER. For example*, C. sakazakii* 767 and 1249, and *E. coli* K1 939 showed major damage to the cells monolayer and both cytotoxic, and in contrast *C. malonaticus* 1569 were low cytotoxic and caused minor damage to the polarized monolayer. This suggesting that bacterial toxicity must be considered in the accurate calculation of attachment, invasion and translocation results.

In general, most of strains linked to meningitis were moderately cytotoxic such as *C. sakazakii* strain 767 and 1249 and *C. turicensis* 564.

With regard to Caco-2 cells, all of *C. sakazakii* displayed moderate cytotoxicity. With exception of NEII strain 703 that showed more than 6-fold increase in the cytotoxicity compared to the blank and ST3 strain 978 that showed about 62-fold increase in the cytotoxicity compared to the blank (Figure 4-16). *C. malonaticus* strain 1569 and *C. condimenti* 1330 displayed 4.5 and 6.2 folds of non-treated cells respectively. High cytotoxic strains such as *C. malonaticus* 1569 and *E. coli* K1 393 were further high translocated strains, and among those who caused major reduction in the TEER mainly with H4 cells, which is in agreement with the suggested role of bacterial toxicity on the other virulence factor including translocation.

This assay led to discover the very high cytotoxic *C. sakazakii* strain 978 from ST3 group. As described in results this strain displayed about 92-fold of the blank. Interestingly, this

strain does not share this level of cytotoxicity with other members of the same group of ST3 stated in figure 4-16.

Therefore, this strain was further investigated to determine the potential reasons behind this high cytotoxicity. The findings suggested that this strain have to be live, active and in direct contact with host cells. This suggests two main factors: firstly, the type of the secretion system such as type VI secretion system (T6SS) and associated proteins such as *vgrG* that thought to be involved in different virulence factors including adherence, invasion, and cytotoxicity (Joseph *et al,* 2012). As this strain needs to be in direct contact with host cells and able to inject the toxic proteins to the host cells. BLAST search indicated that this gene is present in this strain and many other non-cytotoxic strains.

The second possible factor in the high cytotoxicity of this strain is production of toxic compounds such as enterotoxins that can kill and damage the cells. However, incubating of this strain in the apical chamber of 0.4 µm inserts to disrupt direct contact between bacteria and host cells resulted in omitting of the cytotoxicity. In addition, the disruption of producing new proteins using chloramphenicol indicated that this strain to be able to cause this excessive cytotoxicity must produce new proteins during the interacting with host cells.

The high cytotoxicity of *C. sakazakii* ST3 strain 978 may explain the low invasion into both cell lines, because most of the human cells were killed during the attachment assay, and many of them were floating in the infection medium, subsequently discarded with this suspension. Moreover, the invaded bacteria, may damage the cells internally and damage membrane, which consequently allow the gentamicin to enter human cells and kill the inside bacteria.

4.4.1.5.2 LDH assay.

Lactate dehydrogenase (LDH) assay is based on the measurement of LDH leakage into the culture medium, is widely used in cell injury studies as an indicator of the loss of membrane integrity (Castiaux *et al,* 2016). However, the results obtained supported

Trypan blue findings as *C. sakazakii* ST3 strain 978 and *E. coli K1* strain 939 were the most cytotoxic strains investigated for both H4 and Caco-2 cells.

C. sakazakii ST3 strain 978 and *E. coli K1* strain 939 are enteral feeding tube isolates, and with this level of cytotoxicity they could cause severe damage to neonatal mucosa and facilitate bacterial entry to underlying tissues and blood stream. The cytotoxicity of other *Cronobacter* isolates was varied and ranged from 9% by *C sakazakii* strain 563 to 40% by *C. muytjensii* to H4 cell and from 6%-26% to Caco-2 cells by *C. sakazakii* 1 and 513 by which both of them ST8.

4.4.1.5.3 *C. sakazakii* ST3 cytotoxicity associated genes

In order to identify the potential genes responsible for the high cytotoxicity of *C. sakazakii* ST3 strain 978, Whole genome sequencing was performed for this strain and strain 984, which is also an enteral feeding tube isolate, and results compared using webAct software. The 2 strains differ in about 63 genes. Some genes were of known function while other are unknown. Among these different genes, no specific gene was obviously responsible for cytotoxicity, which is suggesting the potential role of the hypothetical proteins encoded genes in this level of cytotoxicity. In addition, this strain was found to have T6SS which might be one of the key factors behind its cytotoxicity. Further investigating about this strain is strongly required, and according to these results, if this strain allowed to enter into host body, and passed the host defence barriers it might cause serious damage to the host tissues including brain and CNS.

4.4.1.6 Cytotoxicity of bacterial OMPs.

Incubation of purified bacterial OMPs with human cell lines showed overt toxicity, and H4 cell line was more sensitive to bacterial OMPs than Caco-2, which has been observed also with live organisms. OMPs of *C. sakazakii* ST3 strain 978 revealed low cytotoxicity to both cell lines, which pportins our previous finding of the crucial role of live, active bacteria and direct contact between this strain and host cells to cause this excessive cytotoxicity rather than an external bacterial structure and secreted cytotoxin.

Although it was reported that *C. sakazakii* can produce outer membrane vesicles containing toxins to effect host cells (Alzahrani *et al,* 2015), this is the first report of investigating the cytotoxicity of OMPs from *Cronobacter* spp. and *E. coli* K1 to H4 cells compared with adult derived cell line. However, Mittal *et al,* (2009) used a newborn rat model to investigate the role of *Cronobacter* spp. OmpA in the disease development and meningitis occurrence. Their results indicate that OmpA plays a vital role in meningitic cases in rat pups, and high levels of LDH were recorded in serum, brain, and intestine of rat pups, and the suggested that this levels of LDH compared with non-treated rats and *OmpA* mutant *Cronobacter* might due to damage to host cells. According to BLAST search, all of investigated *Cronobacter* isolates have gene encoding for OmpA, which is in agreement with the results showed in Omps profiling (Figure 3-18), where most strains showed bands at size of about 37-40 that thought to be OmpA according to Kothary *et al,* (2017).

Profiling of OMPs using SDS-PAGE showed slight variation between strains in the number and size of OMP bands. The number of bands ranged between 9 and 12 and the size was from 10 to75 KDa. These variation on the number and size of the OMPs could be essential factors behind bacterial virulence factors such as attachment and cytotoxicity of bacterial isolates and their OMPs. However, Cytotoxicity of bacterial OMPs displayed different results of live organism with both cell lines. OMPs of *C. sakazakii* 564 ST41 was the most cytotoxic to H4 cells and SDS-PAGE showed that this strain has 9 bands of OMPs by which two of them about 70 and 75 KDa, that also shown by two *C. sakazakii* strains which are the most cytotoxic strain 978 and the meningitic ST4 strain 767.

However, since there was no obvious link between specific OMPs and measurable phenotype, it was impossible to describe specific activities to specific proteins. To better characterise band products the use of Omps specific monoclonal antibodies (Novinrooz *et al,* 2017), or mass spectrometry (MS) could be employed (Horie *et al,* 2016). It is clear that non-viable extracts from bacterial cells can be inflammatory and cytotoxic to both cell lines.

4.4.1.7 Role of human cytoskeleton in bacterial invasion.

Host cell cytoskeletons were previously found to be involved in adhesion and invasion of pathogenic bacteria such as *Salmonella, Shigella, Yersinia* and *Listeria* into host cells (Navarro-Garcia *et al,* 2013; de Souza Santos and Orth, 2015 and Mittal *et al,* 2016). *Listeria* and *Shigella* were able to induce and exploit the actin polymerization to migrate from cell to another. (Cossart, 2000; Cossart and Sansonetti, 2004; Guiney and Lesnick, 2005; Ferreira *et al,* 2006 and Mittal *et al,* 2016).

Eukaryotic cytoskeleton depolymerisation agents such as Cytochalasin D, Nocodazole and colchicine were used to investigate the role of host actin microfilament and microtubule in invasion of strains to both cell lines**.** The effect of human cytoskeleton inhibitors on the invasion of *E. coli* to brain microvascular endothelial (BMEC) cells (Prasadarao *et al,* 1999), *Plesiomonas shigelloides* with Caco-2 cells (Tsugawa *et al,* 2005) and *Pseudomonas aeruginosa* with human middle ear epithelial (HMEECs) cells (Mittal *et al,* 2014) as well as the invasion of *Cronobacter* spp. into human intestinal (INT407) cell line was investigated by Mohan Nair and Venkitanarayanan. (2007), and results was varied from inhibition to enhanced invasion. However, all of these studies used limited number of strains and the last was based on only one strain.

This is the first time that the eukaryotic cytoskeleton inhibitors were used with the H4 cell line to determine the role of human microfilaments (MF) and microtubules (MT) in the invasion of different isolates from the seven species of the genus *Cronobacter* as well as one *E. coli* K1 strain due to the strong association of this serotype with neonatal meningitis. Overall, the data indicates that the invasion of most of strains was largely inhibited more by microtubule (MT) than microfilament inhibitor (MF), suggesting that invasion is more likely MT dependent than MF. The effect of MF inhibitor Cytochalasin D on bacterial invasion to H4 cells was varied and most strains were largely inhibited such as *C. malonaticus* and *C turicensis*, while some strains increase up to 400% as shown by *C. dublinensis* (Figure 4-22). *E. coli* K1 was inhibited by 50%. This finding suggesting the possible specific role of the bacteria in this process.

The MT inhibitor Colchicine reduced the invasion of all bacterial strains to H4 including *E. coli* and *Salmonella* Enteritidis. Conversely, the effect of other MT inhibitor Nocodazole was variable and it is more likely strain dependant. Invasion of *C. sakazakii* and *C. malonaticus* to neonatal H4 cells was reduced by Cytochalasin D and colchicine, whereas the invasion of *C. sakazakii* strain 709 and 767 was increased by Nocodazole. *C. universalis* did not associate with significant infection and was among the lowest invasive stains of H4, however, the invasion of this strains to H4 cells was increased for about 400% when treated with Nocodazole. This finding suggests that the invasion of these strains is likely not cytoskeleton dependant. This increase in bacterial invasion might due to the disruption of tight junctions, and this assay indicated that *Cronobacter* isolates have different strategies to invade their host cells.

In contrast, the effect of eukaryotic cytoskeleton inhibitors on the bacterial invasion to Caco-2 was different to that of H4 cells. Cytochalasin D increased the invasion of most bacterial strains up to fivefold of the untreated cells as shown by *C. sakazakii* strain 709, and only *C. turicensis* and *C. condimenti* were considerably inhibited. Kim and Loessner (2008) reported up to 700% increase of *Cronobacter* invasion to Cytochalasin D treated cells. However, they investigated only one *Cronobacter* spp. Compared with 12 strains in the recent study representing the seven species of the genus *Cronobacter* as well as some of the most clinically significant strains. The increase of bacterial invasion due to disruption of human MF suggests that this proteins play an important role in controlling of the bacterial invasion in adult cells, in contrast to the invasion to neonatal cells, when inhibition of MF by Cytochalasin D resulted in reducing of bacteria invasion.

The effect of Cytochalasin D on the invasion of *E. coli* K1 to Caco-2 cells was similar to that of H4 cell, which is indicates to the possible strain specific role in this process, and the differences in the pathogenicity strategies between different pathogenic bacteria. The maximum reduction of internalised *E. coli* K1 was about 70% by Nocodazole to H4 cells, demonstrating that *E. coli* K1 strains from the serotype have developed different strategies to infect their hosts. However, this need further investigation to confirm or disprove this suggestion. In contrast with H4 cells, the invasion of *C. sakazakii* to Caco-2 was also enhanced by Colchicine for most of *C. sakazakii* strains as well as *C. dublinensis* type species and *C. malonaticus* strain 1569. *C. sakazakii* 701 and the other *Cronobacter* type species isolates were variably inhibited, suggesting strain specificity in mechanisms of invasion.

Nocodazole enhanced the invasion of most *Cronobacter* type species except *C. malonaticus* and *C. dublinensis,* and significantly inhibited the invasion of some *C. sakazakii* strains to H4 cell and enhanced others. This inhibitor was significantly inhibited the invasion of some *C. sakazakii* to Caco-2 cells*,* majority of which are clinically important isolates*.* The two MT inhibitors used in this project Colchicine and Nocodazole displayed different effects in both cell lines. These differences could be the result of the effect of these agents on the rearrangement of human microtubules or on their triggering by the invading bacteria.

The previous finding of Kim and Loesser (2008) has also suggested the possible strain specific role of inhibitors on Caco-2 cell lines and the possible enhancement of *Cronobacter* spp*.* invasion by Cytochalasin D that significantly increased for up to 700%. However, this might because of the disruption of tight junction development in Caco-2 cells (Sanderson *et al,* 1996).

In conclusion, experiments conducted in this chapter showed that there is a clear difference between adult and neonatal cell lines regarding their response to bacterial co-culture, and in most cases bacterial effects were higher with H4 cells than Caco-2 cells. The bacterial strains associated with serious pathology were more virulent to H4 cells than Caco-2 cells especially with attachment and translocation the two virulence factors associated with activation of host immunity as well as blood, cerebrospinal and brain infection that are commonly responsible for neonatal deaths. This supports our hypothesis that bacterial invasion to neonatal tissues is completely different to the adult situation. Therefore, using an adult derived cell lines to investigate neonatal infections may not match the real situation of what happening in the neonatal infection. In addition, Caco-2 cells are derived from 74 years old adult colon cancer, whereas H4 cells were derived from normal intestine of neonate. These two important factors the age and the source of the cell line make H4 cells more appropriate than Caco-2 cells. This fact led us to the hypothesis that using cancer cell lines to investigate human infection, and especially neonatal infection could be wrong choice and might give false results. However, further investigation is required as few published data are available about using of H4 cells in host pathogen interaction.

Chapter 5. Host response and gene expression.

5.1 Introduction.

The Gram-negative *Cronobacter* genus belongs to the family of Enterobacteriaceae and has recently been characterised as an opportunistic food-borne pathogen. These bacteria have been isolated from different types of food including powdered infant formula (Kandhai *et al,* 2004), as well as clinical samples such as blood, urine, cerebrospinal fluid and faeces (Kucerova et al, 2011), and are associated with mortality rates of up to 80%, and about 20% of neurological disorders in survived infected neonates (Caubilla-Barron et al, 2007, Giri et al, 2011).

Cronobacter is involved in inflammatory diseases including meningitis, an inflammation of the brain and spinal membranes (CDC, 2017). Hunter *et al,* (2008) reported that neonatal infection with *C. sakazakii* triggers variable immune responses including production of chemokines and cytokines such as TNF-α and IL-6 (Hunter *et al,* 2008). In tissue culture models, *C. sakazakii* strains stimulate human macrophage cell line U937 to produce different inflammatory mediators including IL-10, TNF-α and IL-6 (Townsend *et al,* 2007b). Moreover, isolates of *Cronobacter* spp. induce iNOS expression in mice and human cells, which possibly increases NO production, and subsequently leads to alterations of tight junctions and programmed cell death or apoptosis (Hunter *et al,* 2009, Emami *et al,* 2012, Liu *et al,* 2012b).

In addition, *C. sakazakii* was described to induce acute intestinal inflammatory responses, and increase IL-8 production by Caco-2 cells (Choi *et al,* 2012). Emami *et al,* (2011b) indicating that macrophages and neutrophils are very important in clearance of *C. sakazakii* at the early stages of infection, and inhibition of these two immune cell types' results in increase of dendritic cells (DC) recruitment to the *C. sakazakii* infected intestine in a mouse model. This process induces secretion of IL-10 and TGF-α by DC and/or epithelial cells due to the interaction between DC and bacteria, and subsequently leads to tight junction disruption (Emami *et al,* 2011a).

Flagella from different *Cronobacter* isolates including *C. sakazakii*, *C. malonaticus, C. turicensis*, *C. muytjensii* and *C. dublinensis* were found to induce production of pro-inflammatory cytokines IL-8 and TNF-α and anti-inflammatory IL-10 in human macrophages (Cruz-Co´ rdova *et al,* 2012). This study showed that production of these cytokines is through activation of TLR5 by bacterial flagella. Outer membrane vesicles (OMV) of *Cronobacter sakazakii* strain 767, the potentially fatal meningitic isolate, induces IL-8 secretion by Caco-2 cells in a dose-dependent manner (Alzahrani *et al,* 2015). Claud *et al,* (2003) compared the production of IL-8 by Caco-2, H4, HT29-cl19A, by temperature sensitive fetal human intestinal (tsFHI) cell lines in response to TNF-α and IL-1β. Their results indicate that H4 cells have a stronger IL-8 response to both stimulants and produce higher IL-8 levels than the other cell lines.

Therefore, understanding the mechanisms of bacterial infection, and cell responses followed by cytokine production is very important, and can contribute to better understanding of host response to external pathogens, and the mechanisms underlying immune disorders.

Interleukin-1 (IL-1) is very important in the activation of the immune system and induction of cytokine and chemokine production, recruitment of neutrophil migration to inflamed sites and protection against pathogenic microorganisms (Fiocco *et al,* 2011; Sahoo *et al,* 2011). Miller *et al,* (2006) proposed that IL-1 receptor (IL-1R) is the key factor in the activation of host response against microbial infection.

IL-6 and IL-8 are mostly produced in response to bacterial infection and induce inflammatory response in diseases including septicaemia (Hack *et al,* 1995), and meningitis (Rusconi *et al,* 1991). However, Christian *et al,* (2011) found that TNF-α induces Caco-2 cells to produce significant amounts of monocyte chemoattractant protein 1 (MCP1) and IL-8 compared with non-stimulated cells, which suggests that different factors can induce host immunity and enhance cytokine production. Several studies reported the role of dysregulated host immunity in systemic infections. An increase in the production of inflammatory mediator s such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-g and TNF-α has been observed in neonates with disorders such as sepsis and NEC (Ng, 2003; Hui *et al,* 2017).

IL-10 is an important immune-regulatory mediator that plays a vital role in control and regulation of pro-inflammatory cytokine production (Sabat *et al,* 2010; Ouyang *et al,* 2011). However, some pathogenic bacteria such as *Staphylococcus aureus* (Li *et al,* 2015) and *Mycobacterium tuberculosis* (O'Leary *et al,* 2011) succeed in escaping host immunity through induction of high levels of IL-10, which subsequently leads to suppression of pro-inflammatory cytokines.

In addition to cytokines and chemokines, transforming growth factor-β (TGF- β) is a very important regulator of host immunity (Yoshimura *et al,* 2010), controlling cell proliferation and differentiation with immunosuppressive functions. Mice lacking TGF-β, develop multiple severe autoimmune disorders and show reduced survival (Weehuizen *et al,* 2012).

Identification of foreign antigens including pathogens and their components by host cells mainly occurs through pattern recognition receptors (PRRs) including Toll-like receptors (TLRs). Activation of these TLRs leads to activation of host immunity and production of inflammatory mediators that aid in pathogen clearance, and regulation of tissue homeostasis. Dysregulated activation of these PRRs can result in inflammatory disorders (Kigerl *et al,* 2014).

Each of these TLRs can detect specific or many stimulant and different TLRs can be activated by the same stimulant. For example, TLR1 and TLR2 detect bacterial lipopeptide ligands while TLR4 is more specific for bacterial LPS in an indirect way through binding with MD-2 protein.

Different bacterial species have been described to activate the human immune response through different TLRs. *E. coli* activates TLR4 and enhances the production of pro-inflammatory cytokines, while depletion of TLR4 in mice results in decreased inflammatory responses to lung infections (Lee *et al,* 2005). Moreover, TLR2 in Chinese hamster ovary (CHO) fibroblasts showed specificity in detection of Gram-positive bacteria and their cell wall components such as heat-killed *Streptococcus pneumoniae* and *S. aureus,* peptidoglycan (PGN) from *S. aureus* (Takeuchi *et al,* 1999; Yoshimura *et al,* 1999), and lipoproteins from *mycobacterium* (Aliprantis *et al,* 1999).

Cronobacter spp. has been shown to induce TLR responses, and Hunter *et al,* (2008) reported upregulation of TLR6 and TLR2 in rat intestinal epithelial cells (IEC-6) infected with *Cronobacter*. They concluded that this pathogen can induce NEC through activation of host TLRs and induce apoptosis due to high expression of nitric oxide (NO) and inflammatory cytokines such as IL-8. Moreover, the incidence of NEC in both human and mice has been correlated to upregulated expression of TLR4 in intestinal tissues in presence of bacterial LPS (Leaphart *et al,* 2007; Meng *et al*, 2015). Hunter *et al,* (2008) reported an increase of IL-6 production and IL6 mRNA expression in response to infection with *Cronobacter* spp., and proposed that this pathogenic bacterium may initiate the host response through increased binding of bacterial LPS to TLR4 in enterocytes (Hunter *et al*2008).

In addition, isolates of *C. sakazakii* were found to activate TLR3 in rats (Sivamaruthi *et al,* 2015), which has been associated with pro-inflammatory and neuroprotective responses (Bsibsi *et al,* 2006; Kim *et al,* 2008). This may explain the association of *Cronobacter* spp. with reported meningitis cases in neonates, especially in premature and low birth weight infants. TLR3 is known to induce the innate immune responses through stimulation of interferon, cytokine and chemokine production (Willmann *et al,* 2010). Prolonged stimulation of host immunity might decrease cell integrity which facilitates bacterial entrance to the nervous system and development of meningitis.

Therefore, the aim of this part of the study was to investigate the ability of different *Cronobacter* isolates and one *E. coli* K1 isolate to activate the immune response in human epithelial cells using an adult cell model represented by Caco-2 cells and an infant model, represented by the non-malignant H4 intestinal epithelial cell line. Here, host responses via production of a wide range of pro-inflammatory and anti-inflammatory cytokines as well as chemokines was quantified using ELISA and multi-plex magnetic bead assays (Luminex). Moreover, in this chapter we investigate the differences between these two cell lines in the expression of human Toll-like receptors (TLR1-10) and NF-κB in response to bacterial infection. Altogether, these differences were evaluated to determine which cell lines is a better model for investigation of host pathogen interaction, especially in diseases related with infants and neonatal infections.

5.2 Material and methods overview

In this chapter H4 cells will be compared with Caco-2 with regard to their response to bacterial co-culturing. Il-8 production was measured using IL-8 ELISA Ready-SET-Go!® (2nd Generation) kit (see section 2.8.7.1.1). Briefly, non-polarised monolayers of both cell lines were treated for three hours with different bacterial isolates, and supernatants were centrifuged to remove cell debris. For polarized monolayers, supernatants were collected 1 and 3h after bacterial infection and processed similarly (section 2.8.7.2). Lipopolysaccharides (LPS) (Sigma, L6529-1MG) and human interleukin-1 beta (IL-1β, Sigma, SRP6169-10UG) were used as positive controls at concentrations of 100 µg/ml and 1 ng/ml, respectively. The multiplexed cytokine and growth factors assays were performed using luminex kit (Invitrogen/Thermo Fisher Scientific). Supernatants were prepared and assays were performed according to manufacturer's instructions, with support from Dr Ian Spendlove, Head of the Tumour Immunology Group at the University of Nottingham.

No statistical analysis between strains was performed for this assay as only one well was used and the results is an average of about 100 analysis of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham Ccity Hospital. However, Paired t test analysis was used to compare results between H4 and Caco-2 cells.

The expression of human Toll-like receptors was investigated after incubation of bacterial isolates with non-polarized monolayers of H4 and Caco-2 cell lines for a period of 3h in tissue culture flasks (surface area 25 cm^2). Cell medium was removed and cells were harvested by scraping into ice cold DPBS, centrifuged, and then lysed using lysis buffer provided with the SV Total RNA Isolation System (Promega). RNA was isolated according to the manufacturer's instructions. cDNA was obtained using iScript™ cDNA Synthesis Kit (Bio-Rad–1708891) and qPCR was performed in the CFX384 Touch™ Real-Time PCR Detection System (Bio-Red) using reagents and parameters described in section 2.8.8.2.1-6.

5.3 Inflammatory response of H4 and Caco-2 cells.

5.3.1 IL-8 production by non-polarized cells in response to bacterial co-culture

Interleukin 8 production assays were performed using supernatants of bacterial-infected H4 and Caco-2 cells after 3 hours of incubation (Steiner *et al,* 2000) to investigate their inflammatory response. Twenty five *Cronobacter* isolates and the *E. coli* K1 strain were used in this assay. Supernatant collection (Section 2.8.7.2.1), and cytokine measurements were conducted as recommended by company (section 2.8.7.2.2), and IL-8 levels were calculated from standard curves and expressed as picogram per millilitre (pg/mL).

All strains stimulated the production/secretion of detectable quantities of IL-8 in both cell lines. H4 cells exhibited increased inflammatory response to most of the investigated strains ranging from 221 pg/ml induced by the *C. sakazakii* ST8 strain 513 to 4883 pg/ml for *C. sakazakii* ST4 strain 767, which is thought to be responsible for a case fatal meningitis during the French outbreak in 1994 compared with 59pg/ml for untreated cells, a 4 and 76-fold increase compared to untreated control cells, respectively (Figure 5-1).

In general, IL-8 secretion by H4 cells was notably higher in the presence of *C. sakazakii* strains than other investigated species, and ST4 strains were the most effective isolates as shown for strain 767 (4883 pg/ml) and 701 (3145 pg/ml). Moreover, strains isolated from infants with serious pathologies such as necrotising enterocolitis (NEC) and fatal infant infection (FII) stimulated human cells to produce IL-8 ranging from 860 pg/ml to 3000pg/ml which is considerably higher compared to level induced by bacterial LPS and IL-1β ($P<0.05$-0.0001; ANOVA).. *C. sakazakii* ST13 strain 693 and ST31 strain 1249, NECII and fatal infant infection isolates induced IL-8 production in H4 cells at levels of 2555 and 2960 pg/ml. Other *Cronobacter* isolates enhanced IL-8 production. Conversely, IL-8 produced in response to *C. malonaticus* isolates ranged from 517 to 1500 pg/ml. *C. malonaticus* ST 307 strain 1569, the first recorded isolate associated with neonatal fatal meningitis induced IL-8 secretion more than 4-fold (1000 pg/ml) compared to the *C. sakazakii* strain 767 which is associated with same disease.

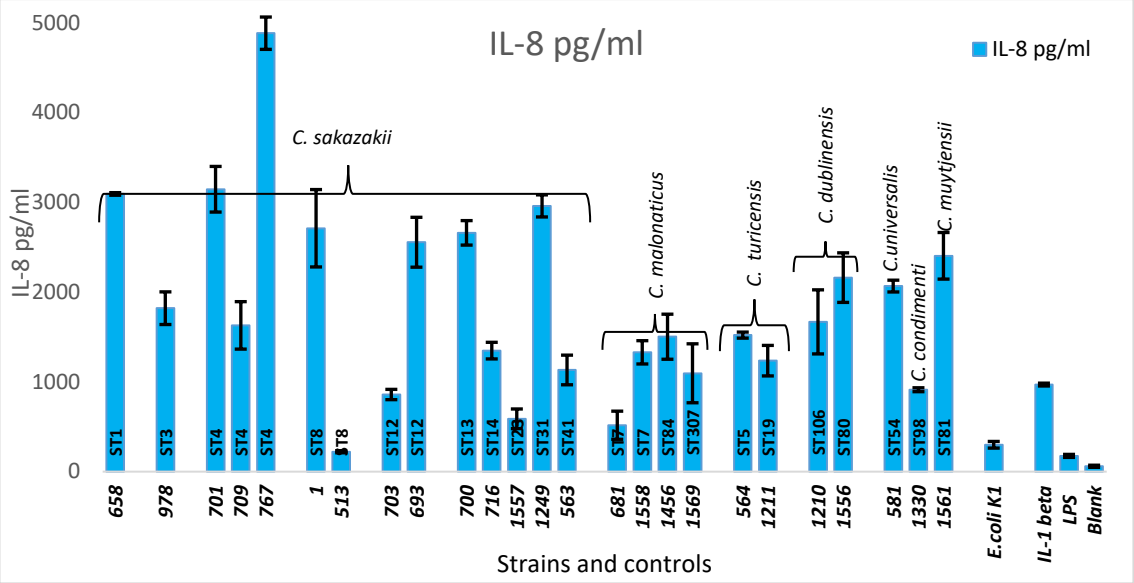


Figure 5-1 Bacterial isolates induced interleukin 8 (IL-8) production in H4 cells. H4 cells were co-cultured with bacterial isolates for 3h, supernatants were collected, centrifuged and IL-8 levels were determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. Strain 513 is non-motile and showed low IL-8 induction, indicating the possible role of flagella and/or pili in host response. Data are represented as mean ± SEM from three independent experiments performed in triplicates. Information inside the columns indicate strains sequence type (ST). Most strains showed significant differences of the mean values of IL-8 production induced by the strains compared to LPS or IL-1 beta that were positive controls in this assay (P<0.05-0.0001; ANOVA). No significant differences were shown by strains 513, 1557, 681 and *E. coli* K1 when compared with bacteria LPS.

Two *C. turicensis* isolates were investigated and have been chosen according to their clinical history. Strain 564 is a blood isolate from neonatal fatal meningitis and 1211 is a clinically important fatal infant infection (FII) isolate. These two strains can be considered as a moderate IL-8 inducers with levels of 1550 and 1230 pg/ml, respectively. With regard to *C. dublinensis* isolates 1210 (ST106) the type species strain at NTU collection (non-clinical) and 1556 ST80 which is an abscess - base of spine isolate, induced IL-8 secretion higher than that induced by *C. malonaticus* and *C. turicensis* isolates which are, 1700 and 2170 pg/ml, respectively. The other three *Cronobacter* isolates represented the last three species of this genus and are non-clinical isolates. These strains, *C. universalis, C. condimenti* and *C. muytjensii,* and induced 2067, 912 and 2400 pg/ml respectively.

Strains from same ST group can enhance the inflammatory response differently, for example, *C. sakazakii* ST4 strains exhibited high variation in cell stimulation, as ST8, ST12 and *C. malonaticus* ST7 (figure 5.1). H4 cell line showed weak inflammatory response

towards *E. coli* K1 strain 939 with IL-8 production of only 299 pg/ml. This may be a strategy developed by this isolate to escape host response, as among *E. coli* species of this particular serotype are the most associated with neonatal meningitis.

Human IL-1 beta, the positive control, enhanced moderate response with about 1000 pg/ml, while bacterial LPS which is used as a secondary positive control in this assay showed considerably lower IL-8 production by H4 cells (175 pg/ml), while cells incubated with only medium without FBS produced only 60 pg/ml.

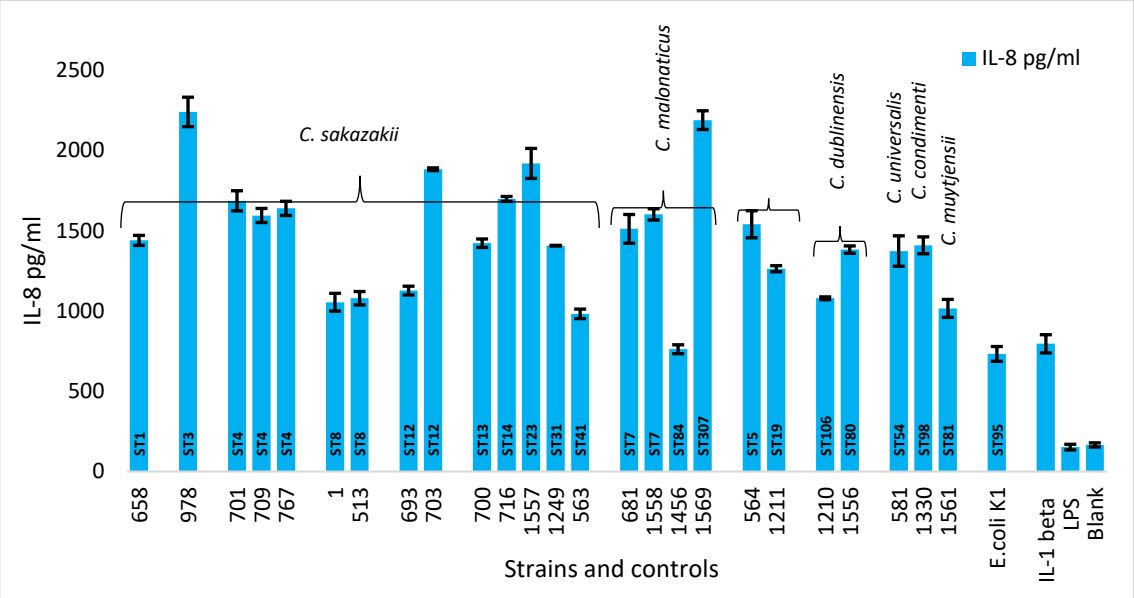


Figure 5-2 Bacterial isolates induced interleukin 8 (IL-8) production in Caco-2 cells. Caco-2 cells were co-cultured with bacterial isolates for 3h, supernatants were collected, centrifuged and IL-8 levels were determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. strain 513 is non-motile and showed low IL-8 induction, indicating the possible role of flagella and/or pili in host response. Data are represented as mean ± SEM from three independent experiments performed in triplicates. Information inside the columns indicate strains sequence type (ST). Most strains showed significant differences with LPS, and only strains 563, 1546 and *E. coli* K1 strain 939 were failed to onduce significantly different levelsof IL-8 when compared with IL-1 beta and P value wa varied (P<0.05-0.0001; ANOVA)

With regard to Caco-2 cells, IL-8 production was considerably lower than that observed in H4. The maximum concentration detected was 2240 pg/ml in response to *C. sakazakii* ST3 strain 978 (Figure 5-2), which is originally an enteral feeding tube (EFT) isolate which is significantly higher than the positive controls LPS and IL-1β (P<0.0001; ANOVA). Caco-2 response to the investigated strains was different to H4 cells. For example, IL-8 production in response to *C. sakazakii* ST4 strains 701, 709 and 767 was approximately the same (1600-1685 pg/ml), although they were isolated from different neonates with

different symptoms, where two of them died due to either NECIII (701) or meningitis (767). Similarly, ST8 isolates 1 and 513 had similar effects (1050 pg/ml), though they are clinically different and strain 513 was non-motile. In H4 cells IL-8 production in response to 513 was significantly lower compared to the motile strain 1. However, most of the clinically important strains induced significantly higher IL-8 in H4 than Caco-2 cells ($P < 0.05$/Paired t test).

Strains from ST12 induced different IL-8 levels, with cells exposed to the asymptomatic isolate 693 producing 1200 pg/ml, whereas NECII isolate strain 703 caused more IL-8 production at 1900 pg/ml. Cell stimulation by *C. malonaticus* varied and IL-8 produced as a response to strains from ST7 was similar. Strain 1569 (ST307) resulted in IL-8 secretion at 2190 pg/ml which was the highest among *C. malonaticus* strains and about twofold higher than that produced by H4 in presence of same strain. The IL-8 production by Caco-2 cells after 3h incubation with *C. turicensis, C. dublinensis, C. universalis, C. condimenti* and *C. muytjensii* ranged from about 1000 to 1540 pg/ml which is about 6-10 fold higher compared to unstimulated cells. Caco-2 cells showed more sensitivity to *E. coli* K1 than H4 cells, as the produced IL-8 was about 2.5-fold higher than that produced by H4 cells. This could be one of the mechanisms of how this strain escapes the neonatal immune system and invade the blood brain barrier (BBB).

5.3.2 IL-8 production by polarized cells in response to bacterial infection

Human epithelial cell lines H4 and Caco-2 were further investigated with regard to the inflammatory response dependence on the site of stimulation. Thirteen bacterial isolates were chosen according to their clinical history and our findings from the non-polarized assay. These strains are: nine *Cronobacter* isolates, the *E. coli* K1 isolate, and *Salmonella Enteritidis* and *E. coli* K12 as positive and negative controls, respectively.

Interestingly, for both cell lines when bacteria were added to the to the upper chamber, after one hour incubation IL-8 secretion was much higher than that detected in the basolateral chamber ($P < 0.001$). *C. sakazakii* ST13 strain 693, originally isolated from an asymptomatic neonate showed the highest amount of IL-8 in the upper chamber (680pg/ml) compared with only 116 pg/ml in the basolateral side (Figure 5.3). Generally,

the highest amounts of IL-8 secreted in the upper chambers were induced by strains isolated from neonates suffering from serious infections such as meningitis and fatal infections. In contrast, *C. turicensis* strain 564, a meningitic isolate, displayed the lowest stimulation in H4 cells among *Cronobacter* isolates, with only 86 pg/ml IL-8 secreted after 1 hr incubation with the bacteria. IL-8 secreted in response to *E. coli* K1 was 518 pg/ml compared with only 33 pg/ml in response to the non-pathogenic *E. coli* K12 (15-fold lower).

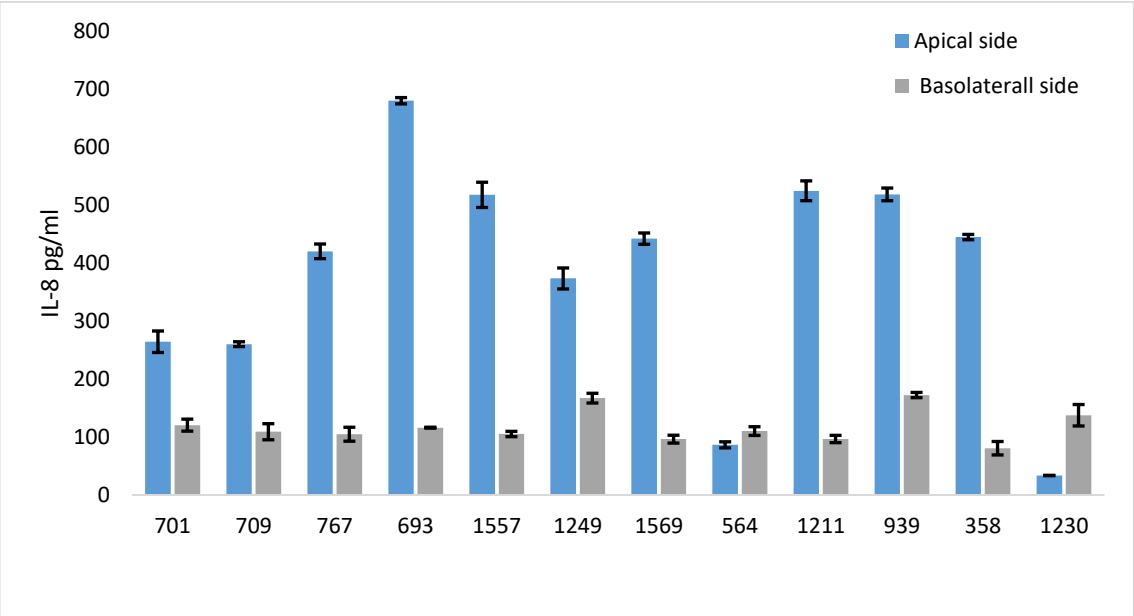


Figure 5-3 IL-8 secretion in upper and basolateral chambers by polarized H4 cells. Bacterial isolates induced the secretion of different levels of interleukin 8 (IL-8) depending on the site of stimulation. Bacterial isolates were added to the upper chamber of polarized H4 cells and incubated for 1h. Then, 100µl of the supernatants were collected, centrifuged, and IL-8 was determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. Data are represented as mean ± SEM from three independent experiments performed in triplicates.

After three hours incubation, IL-8 production was significantly increased in both chambers ($P < 0.01$/ Paired t test), and some strains showed higher stimulation of IL-8 production in the basolateral chamber more than the apical chamber. IL-8 production in response to *C. sakazakii* ST4 strains 701 and 709, and ST12 strain 693 in the upper chamber was 855, 1109 and 1557 pg/ml compared with 430, 870 and 1080 in the lower chamber, respectively. Likewise, the recovered IL-8 from the basolateral chamber after 3h incubation with *E. coli* K1, *S. Enteritidis* and *E. coli* K12 was more than that recovered from the upper side. In contrast, for some of *Cronobacter* isolates more IL-8 was detected in the upper chamber including for the three meningitic isolates; *C. sakazakii*

ST4 strain 767 (French outbreak), *C. turicensis* ST5 strain 564 and *C. malonaticus* ST307 strain 1569 (Figure 5.4).

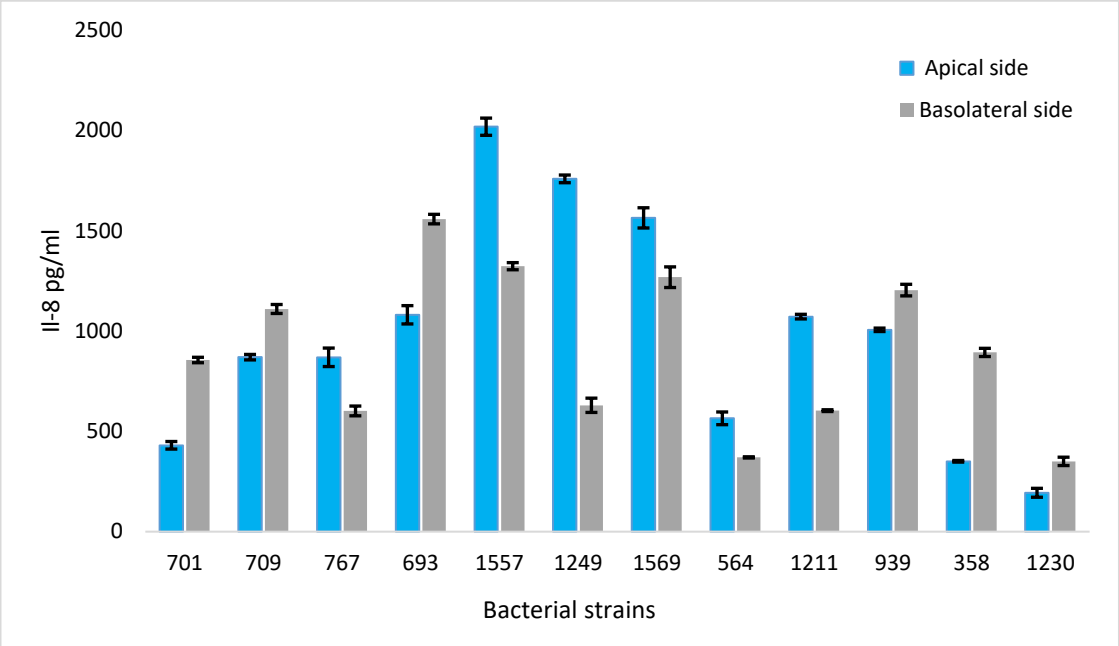


Figure 5-4 IL-8 secretion in upper and basolateral chambers by polarized H4 cells. Bacterial isolates induced the secretion of different levels of interleukin 8 (IL-8) depending on the site of stimulation. Bacterial isolates were added to the upper chamber of polarized H4 cells and incubated for 3h. Then, 100µl of the supernatants were collected, centrifuged, and IL-8 was determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. Data are represented as mean ± SEM from three independent experiments performed in triplicates.

With respect to Caco-2 cells, although cells exhibited an increase in the amount of IL-8 produced between 1hr, (figure 5-5) and 3h, (figure 5-6) in both upper and basolateral sides, the levels of interleukin remained higher in the upper chamber compared to the lower one. Interestingly, *C. sakazakii* ST13b strain 693 was the most stimulative strain at 3h, with IL-8 secretion around 2000 pg/ml. At this time point, strains isolated from neonates with severe meningitis caused Caco-2 cells to produce high amounts of IL-8 with an average of 1725 pg/ml.

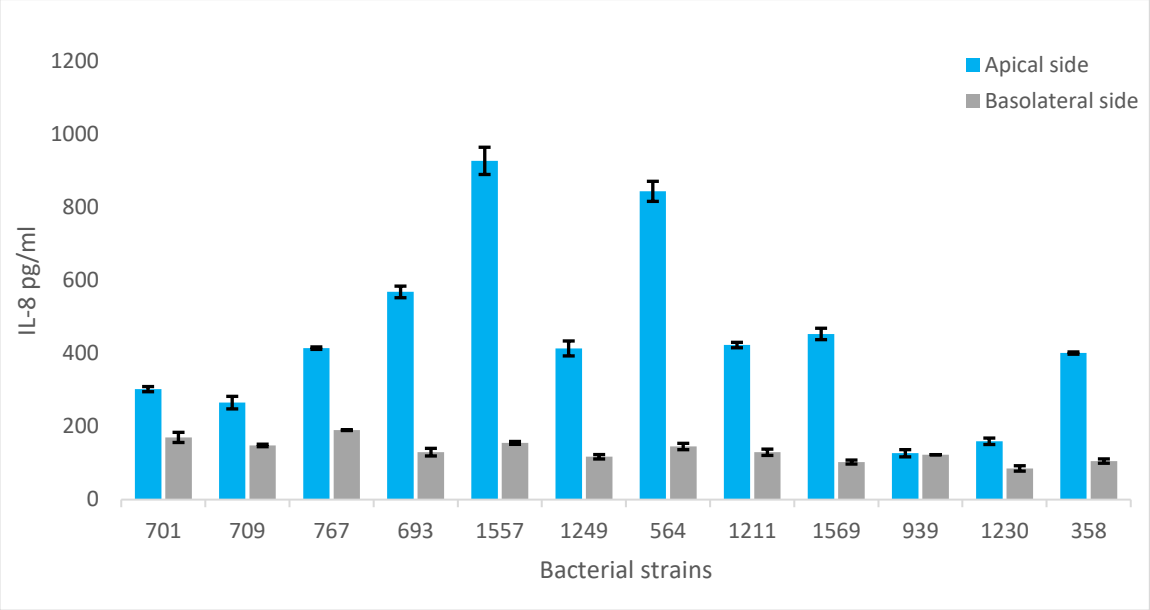


Figure 5-5. Secretion in upper and basolateral chambers by polarized Caco-2 cells. Bacterial isolates induced the secretion of different levels of interleukin 8 (IL-8) depending on the site of stimulation. Bacterial isolates were added to the upper chamber of polarized Caco-2 cells and incubated for 1h. Then, 100µl of the supernatants were collected, centrifuged, and IL-8 was determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. Data are represented as mean ± SEM from three independent experiments performed in triplicates.

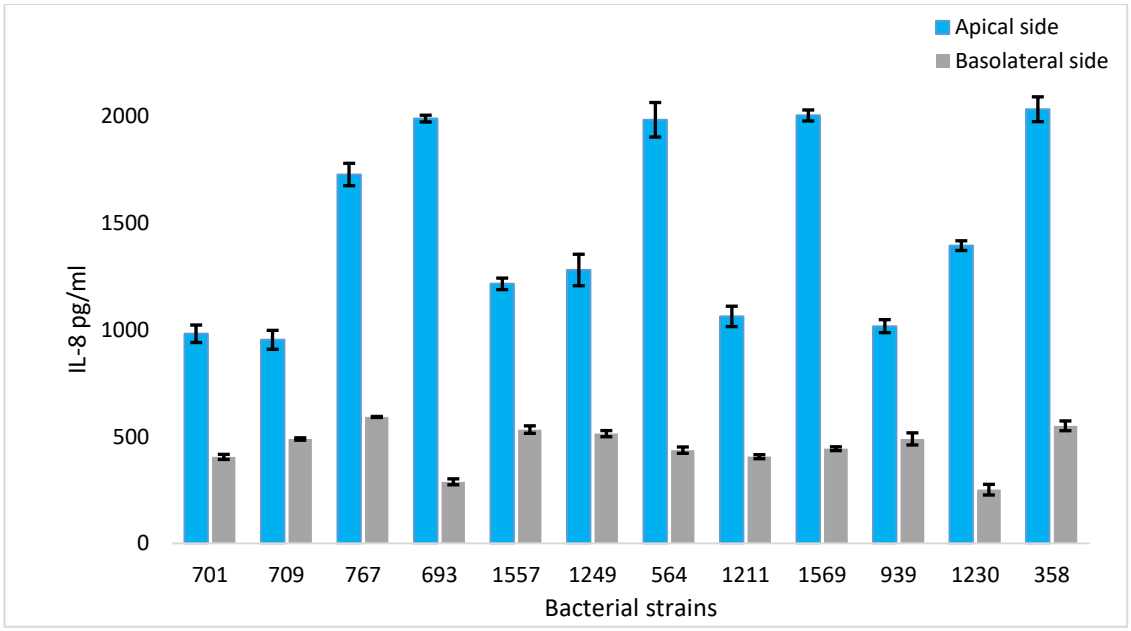


Figure 5-6. IL-8 secretion in upper and basolateral chambers by polarized Caco-2 cells. Bacterial isolates induced the secretion of different levels of interleukin 8 (IL-8) depending on the site of stimulation. Bacterial isolates were added to the upper chamber of polarized Caco-2 cells and incubated for 3h. Then, 100µl of the supernatants were collected, centrifuged, and IL-8 was determined using ELISA Ready-SET-Go! (2nd Generation) kit. Results are expressed as pg/ml. Data are represented as mean ± SEM from three independent experiments performed in triplicates.

5.3.3 IL-8 production by H4 and Caco-2 cells in response to bacterial OMP.

Bacterial outer membrane proteins (OMPs) stimulated both cell lines to produce detectable amounts of pro-inflammatory interleukin 8 (IL-8). H4 cells were more responsive to OMPs and released more IL-8 than Caco-2. Both cell lines secreted lower IL-8 levels in response to the OMPs than that induced by live bacteria (P<0.0001/Paired t test). IL-8 produced by H4 cells in response to OMPs from *C. sakazakii* ranged from 960 pg/ml to 2060 pg/ml, and OMPs of *C. sakazakii* ST 31 strain 1249 induced the highest IL-8 secretion, while the lowest was obtained from strain 1557 (P<0.0001/ Tukey). There was no significant difference between the effect of OMPs from the high cytotoxic and non-cytotoxic ST3 strains 978 and 984 (1350 pg/ml and 1270 pg/ml, respectively). A small difference was observed between ST8 isolates. OMPs from strain 513 induced lower levels than those from strain 1, similar to the results obtained for live organisms. OMPs of *C. malonaticus* strain 1569, the only recorded meningitic isolate from this species exhibited the lowest stimulation of H4 cells. Moreover, low IL-8 production was observed in response to *C. turicensis* strain 1211 and *E. coli* K1 strain 939.

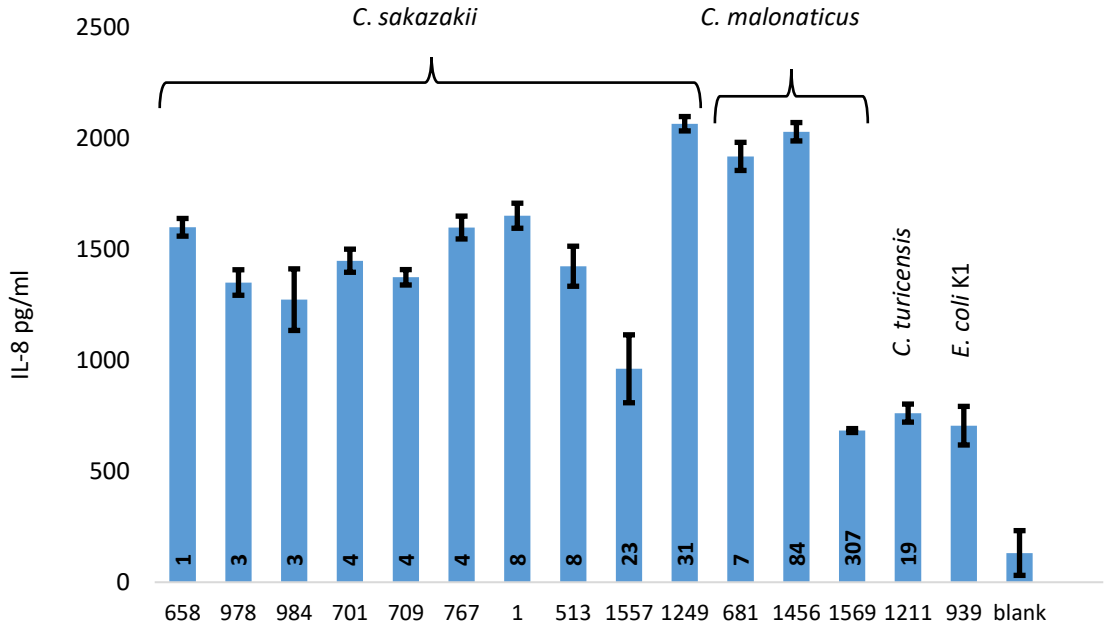


Figure 5-7 Bacterial OMPs induce IL-8 production in non-polarized H4 cell line. Purified OMPs were incubated with human cells for 3h at a concentration of 100 µg/ml of protein. Supernatants were collected, centrifuged and IL-8 was quantified using ELISA. Numbers in the columns indicate bacterial ST.

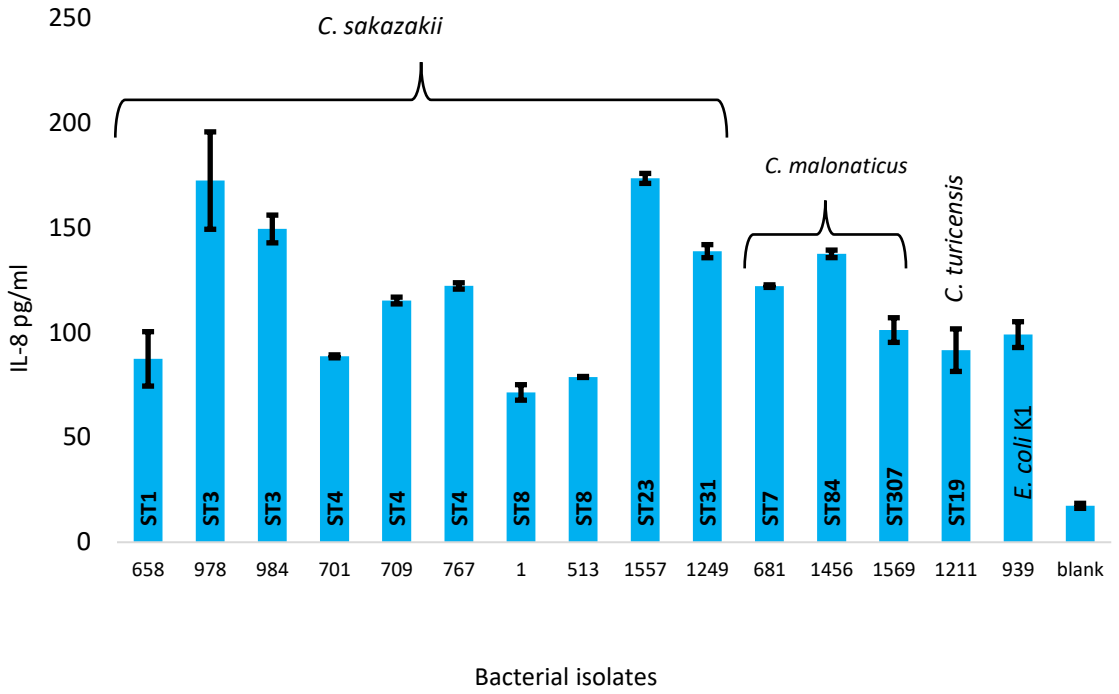


Figure 5-8 Bacterial OMPs induce IL-8 production by non-polarized Caco-2 cell line. Purified OMPs was incubated with human cells for 3h at a concentration of 100 µg/ml of protein and then supernatant collected, centrifuged and il-8 quantified using ELISA method. Numbers in the column indicate bacterial ST

With regard to Caco-2 cells, bacterial OMPs induced significantly lower IL-8 secretion than H4 cells (P<0.0001/Paired t test). For example, OMPs from *C. sakazakii* ST1 strain 658 resulted in IL-8 secretion about 18 times lower and OMPs from the meningitic ST4 *C. sakazakii* strain 767 about 13-fold lower in caco-2 than in H4 cells (Figure 5.8). Among *C. malonaticus* isolates, ST84 strain 1546 induced the highest IL-8 secretion by H4 and Caco-2 cells (2029 pg/ml and 137 pg/ml, respectively). *C. sakazakii* ST3 and ST23 strains 978 and 1557 respectively were the stronger stimulants of Caco-2 cells (P<0.0001/Tukey), also observed for live bacteria (figure 5-2). These results provide a possible evidence that bacterial OMPs play important roles in the stimulation of host immune response and induction inflammatory cytokine production. Moreover, neonatal epithelial cells exhibited increased responsiveness to bacterial OMPs compared with adult cells. This finding suggests the high sensitivity of neonatal immune system to external antigens and limited control over inflammation. This finding supports the use of the H4 cell line and other neonatal cells as the appropriate tool to investigate host pathogen interactions in inflammatory diseases in neonates.

5.3.4 Activation of immune responses of the intestinal epithelial cells by selected bacterial isolates.

Pro-inflammatory response is a very important mechanism of the host innate immune system as defence against pathogenic microorganisms, and consequently plays an important role in the early identification and induction of the suitable response to the invading pathogens. The intestinal epithelial cells are the first contact line with both pathogenic and commensal flora in gastro intestinal tract, and provide a protective barrier from infectious microorganisms coming from the gastrointestinal tract. Therefore, the secretion of different cytokines and growth factors in response to bacterial infection was assessed using Bio-Plex ELISA. EGF, IFN-γ, IL-1β, IL-10, IL-12, IL-2, IL-4, IL-6, IL-8, IP-10, MCP-1 and TNF-α levels were analysed in cell supernatants in response to thirteen bacterial isolates including 12 *Cronobacter* isolates: 8 *C. sakazakii* isolates, 3 *C. malonaticus* isolates, 1 *C. turicensis* isolate, and one *E. coli* K1 isolate. Lipopolysaccharides from *E. coli* (LPS) and human IL-1 beta were used as positive controls in this assay, and fresh medium as a negative control.

The ability of bacterial isolates and positive controls to trigger the secretion of tested cytokines varied. There was a clear difference between the two cell lines in the responses to the investigated stimulants. Some cytokines were below detectable levels in the samples and considered to be negative. IFN-γ and IL-2 were undetectable in all samples and controls with exception of *C. malonaticus* ST7 strain 681 which induced IL-2 production (36 pg/ml) in H4 cells (Figure 5.9). TNF-α secretion was only induced in H4 cell by LPS and human IL-1β. Moreover, some markers such as IL-12 were undetectable in the samples obtained from Caco-2 cells, and only detected in some samples collected from H4 cells.

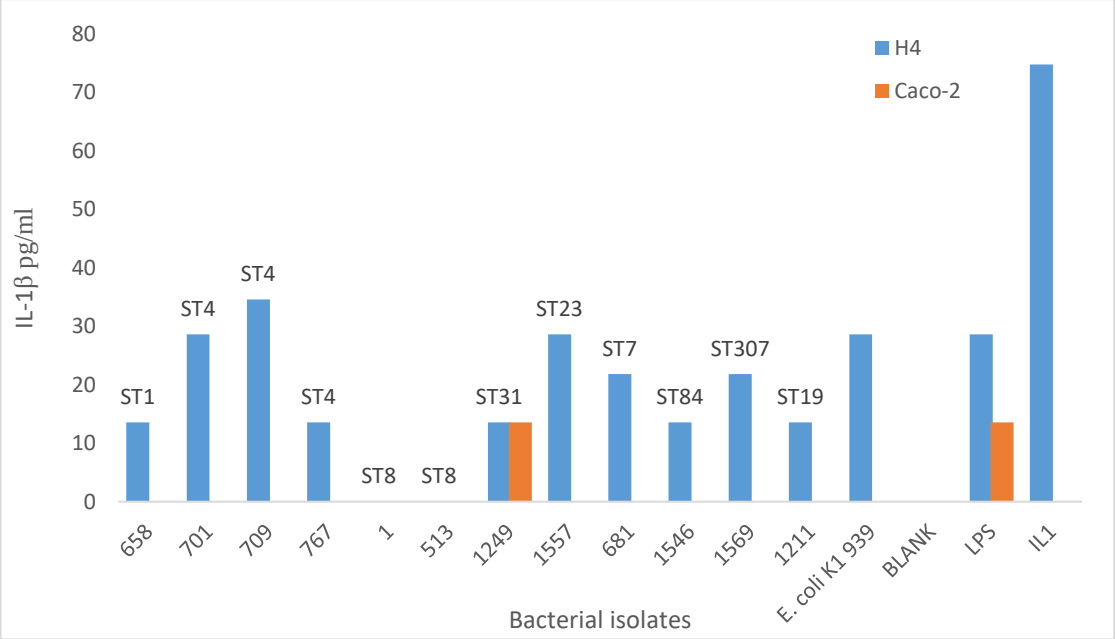


Figure 5-9. Bacteria induced IL-1β expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, IL-1β levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital

Most of the bacterial isolates induced IL-1β secretion in H4 cells, except *C. sakazakii* ST8 strains 1 and 513 where IL-1β was not detected, while only *C. sakazakii* ST31 strain induced Caco-2 cells to produce 13 pg/ml. In H4, secreted IL-1β ranged from 13.5 induced by *C. sakazakii* strains 658, 767, 1249, *C. malonaticus* strain 1546 and *C. turicensis* strain 1211 to 34 pg/ml by *C. sakazakii* ST4 strain 709. However, the cell lines also differed in their response to the positive controls, with the highest amount of IL-1β induced by H4 cells (74 pg/ml) when stimulated with human IL-1β that showed negative results with Caco-2 cells. H4 cells were more inducible by bacterial LPS than Caco-2 cells.

With regard to IL-6, which is a pro-inflammatory cytokine, H4 and Caco-2 cells displayed different responses to the selected bacterial isolates and positive controls. Most of the investigated strains (9/13) induced moderate levels of IL-6 secretion in H4 cells (1000-1500 pg/ml), while *E. coli* K1 and *C. sakazakii* ST23 strain 1557 caused the highest response among strains (>2500 pg/ml).

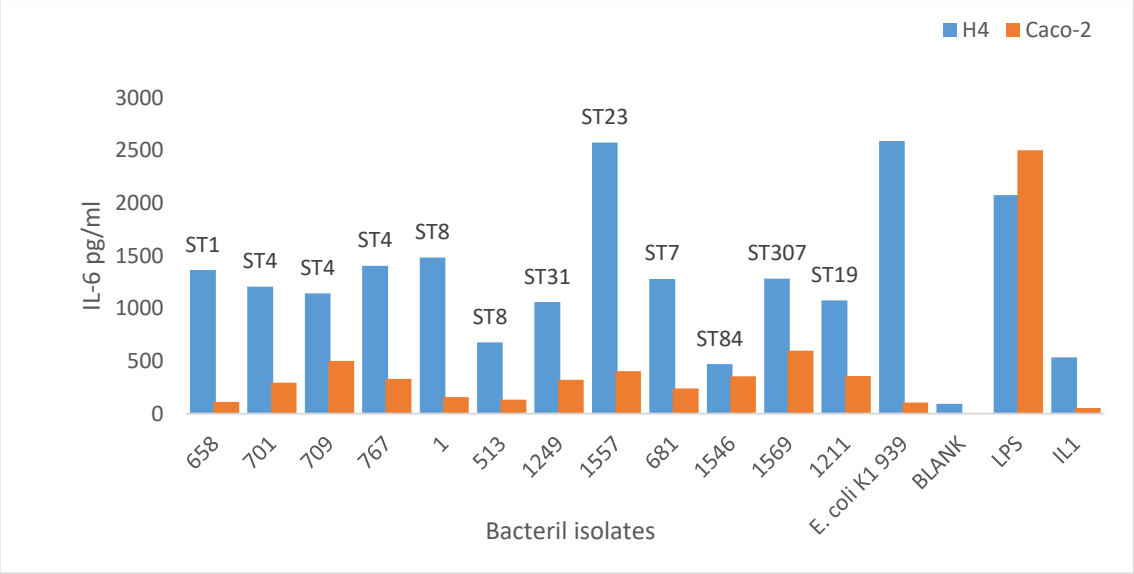


Figure 5-10. Bacteria induced IL-6 expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, IL-6 levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

Conversely, all strains showed low level of IL-6 induction in Caco-2 cells, with the maximum induction by *C. malonaticus* 1569 (599 pg/ml) (Figure 5. 10). *C. sakazakii* ST1 strain 658, and ST8 strains 1 and 513 as well as *E. coli* K1 induced the lowest levels of IL-6 secretion (<160 pg/ml). Caco-2 were more responsive to LPS than H4 cells (2000 and 2500 pg/ml respectively), while less responsive to IL-1 beta (56pg/ml and 535 pg/ml, respectively).

Bacterial isolates induced the secretion of high levels of interleukin 8 (IL-8) mainly in H4 cells (Figure 5. 11). Cell lines exhibited high variation in their responses to all stimuli including the positive controls. Moreover, there have been a considerable variation in the level of induction between strains from same species and strains belonging to the same sequence type. *C. sakazakii* ST8 strain 1 showed 2-fold higher induction than strain 513 from the same ST group. Regarding the response of H4 cells to *Cronobacter* strains, the highest levels of IL-8 were induced by *C. sakazakii* ST23 strain 1557 (14700 pg/ml), which was the most invasive strain in H4 cells, followed by *C. sakazakii* ST4 strain 701 that is linked to neonatal death due to severe NECIII in the French outbreak of 1994 (Figure 5-11).

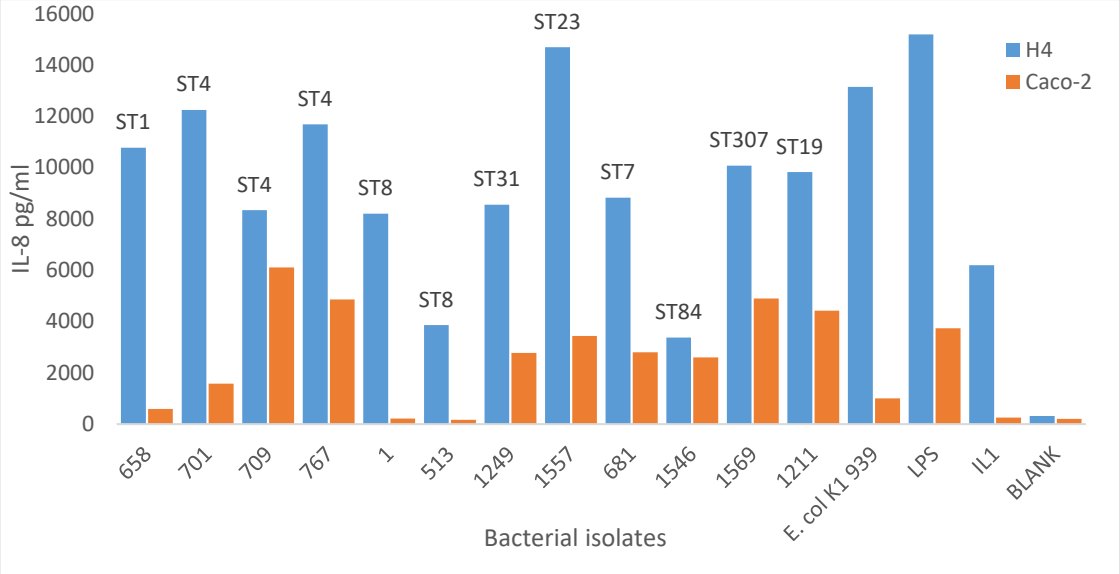


Figure 5-11. Bacteria induced IL-8 expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, IL-8 levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

E. coli K1, the second stronger stimulatory strain, induced IL-8 production in H4 at 13150 pg/ml. In contrast, Caco-2 cells showed maximum IL-8 induction of 6113 pg/ml by *C. sakazakii* ST4 strain 709, isolated from an infant with septicaemia in French outbreak in 1994, compared with 8343 pg/ml showed by H4 in response to same strains, followed by strain 767 from same ST group (Figure 5-13). Some strains such as *C. sakazakii* ST4 strain 701 and *E. coli* K1 induced IL-8 in H4 cells 8 to 13-fold higher compared to Caco-2 cells. In addition, H4 cells exhibited significantly higher IL-8 production ($P<0.0001$) in response to IL-1β and bacterial LPS than Caco-2 cells (Figure 5-11).

The stimulation of interferon gamma (IFN)-g induced protein 10 (IP-10) secretion by the selected bacterial isolates was also investigated. However, the amount of produced IP-10 was very low compared to IL-8 and IL-6. H4 cells were more responsive to bacteria and bacterial LPS than Caco-2 cells. Results varied, and no correlation was evident between IP-10 secretion and clinical history or sequence type. *E. coli* K1 strain 939 showed the highest level of induction, followed by *C. malonaticus* ST7 strain 681. In general, other bacterial strains showed low variation in induced IP-10 with concentrations ranging from 0.86 to 1.13 pg/ml (Figure 5.12).

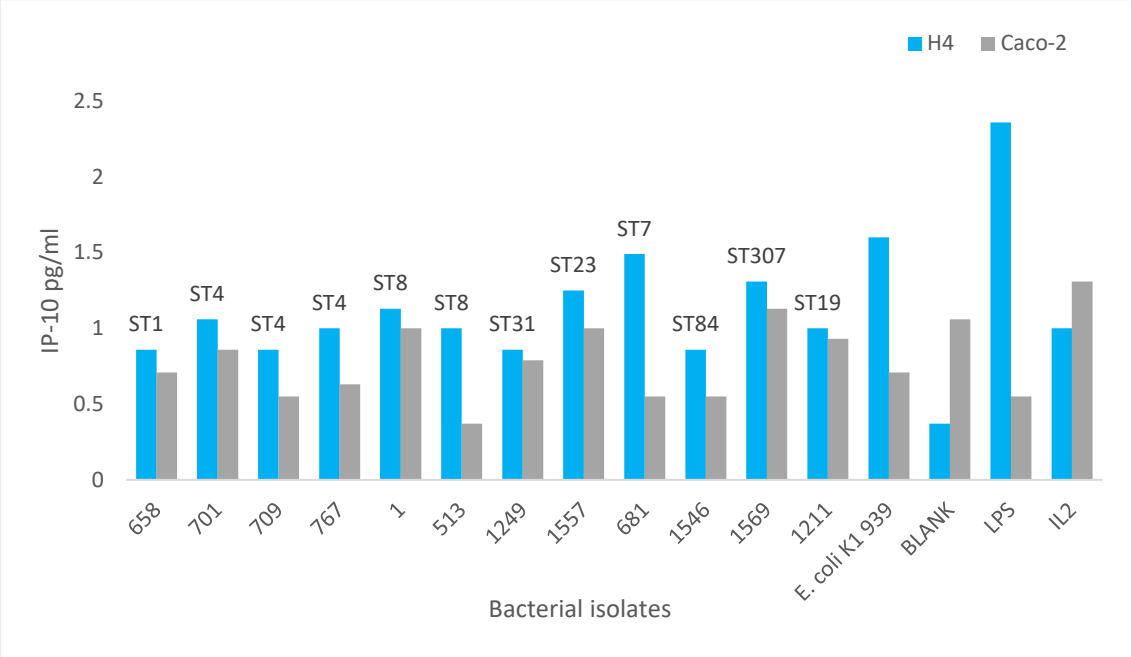


Figure 5-12 Bacteria induced IP-10 expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, IP-10 levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

With regard to Caco-2 cells, only *E. coli* K1, *C. sakazakii* 513 and *C. malonaticus* 681 exhibited considerable differences compared to those in H4, while minor differences were observed for other strains. Positive controls had different effects on the cell lines. LPS induced H4 to produce four times IP-10 higher than Caco-2 cells, whereas IL-1β caused Caco-2 cells to produce IP-10 slightly more than H4 cells ($P < 0.05$/ paired t test) (Figure 5-12).

Monocyte chemoattractant protein 1 (MCP-1) is one of the significant chemokines that regulate migration of monocytes/macrophages to the site of site of tissue injury or infection. (Deshmane *et al,* 2009), and plays an important role during intestinal inflammation especially in inflammatory bowel disease (IBD) (Kucharzik *et al,* 1998). All of the tested strains showed moderate induction of MCP-1 in H4 cells from 165-287 pg/ml, 314 pg/ml were produced in response to bacterial LPS and 214 pg/ml by human IL-1β. Interestingly, *C. sakazakii* ST8 did not induce this chemoattractant in the adult derived epithelial cell line Caco-2 cells, which supports the hypothesis of association of this serotype with neonatal NEC due to the immaturity of neonatal intestine (Figure 5-13).

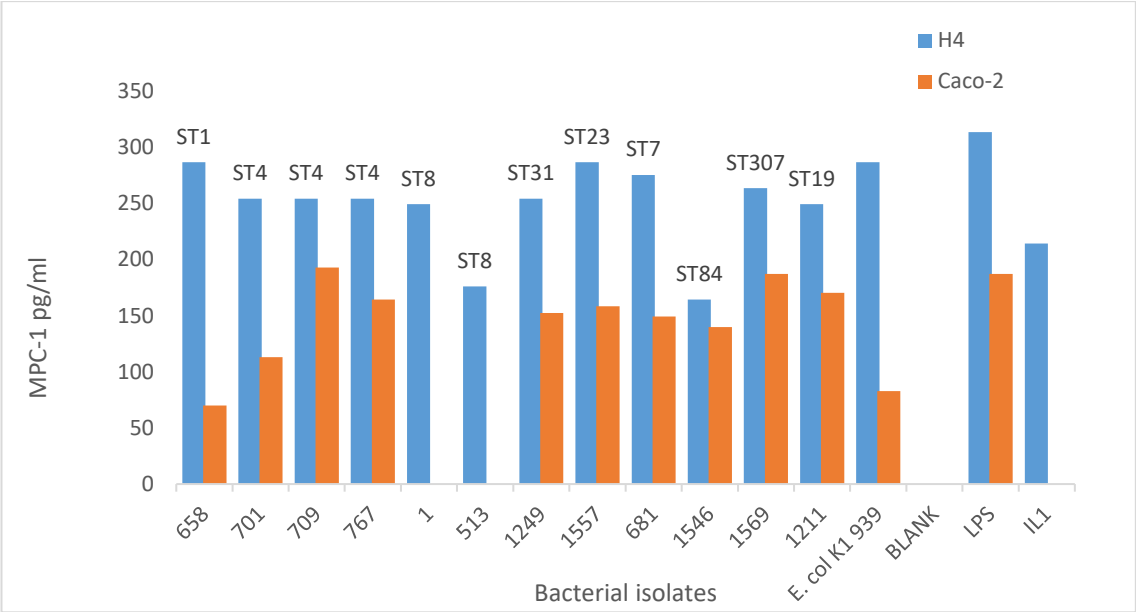


Figure 5-13 Bacteria induced MPC-1 expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, MPC-1 levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

Though, *E. coli* K1 was among the most effective stimuli for H4 cells, it was the second least effective stimulus for Caco-2. Additionally, human IL-1β had no effect on Caco-2 cells compared with MCP-1 production induced in neonatal H4 cells (214 pg/ml).

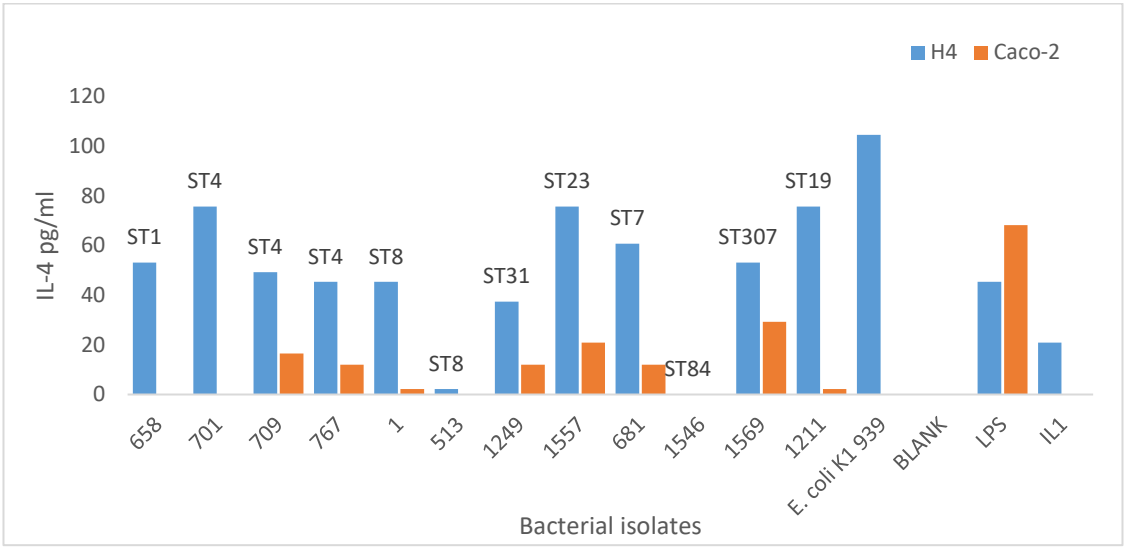


Figure 5-14 Bacteria induced IL-4 expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, IL-4 levels were determined using Bio-Plex ELISA. No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyses of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

Bacterial isolates were also investigated in respect to their ability to induce the secretion of anti-inflammatory cytokines such as IL-4 and IL-10. IL-10 was undetectable in both cell lines over three independent experiments upon either bacterial stimulation or treatment with positive controls. *E. coli* K1 caused the maximum IL-4 secretion (104.6 pg/ml) in H4 cells, while the concentrations produced in Caco-2 cells were below the detection levels of this kit (<1 pg/ml) (data not shown). *C. sakazakii* ST8 strains 1 caused IL-4 induction by H4 cells more than that noted by 513, where motile strain 1 induced about 45 pg/ml whereas strain 513 only 2 pg/ml (Figure 5-14). Among *C. sakazakii* ST4 isolates (701, 709 and 767), strain 701 linked to neonatal death due to NECIII, displayed a IL-4 induction of 75.6 pg/ml, comparatively higher than other strains from same sequence type. Similar results were obtained by *C. turicensis* type species strain which was also isolated from fatal infant infection, and *C. sakazakii* ST32 strain 1557 (Bronchial secretion). Only *C. malonaticus* strain 1546 did not show any effect on IL-4 levels in H4 cells. IL-1β and about 40% of bacterial isolates had no effect on IL-4 secretion in Caco-2 cells, and 23% of isolates induced approximately 2 pg/ml, with the highest at 29 pg/ml by *C. malonaticus* strain 1569. Interestingly, Caco-2 cells were more responsive than H4 cells to bacterial LPS in regard to IL-4 production (Figure 5-14).

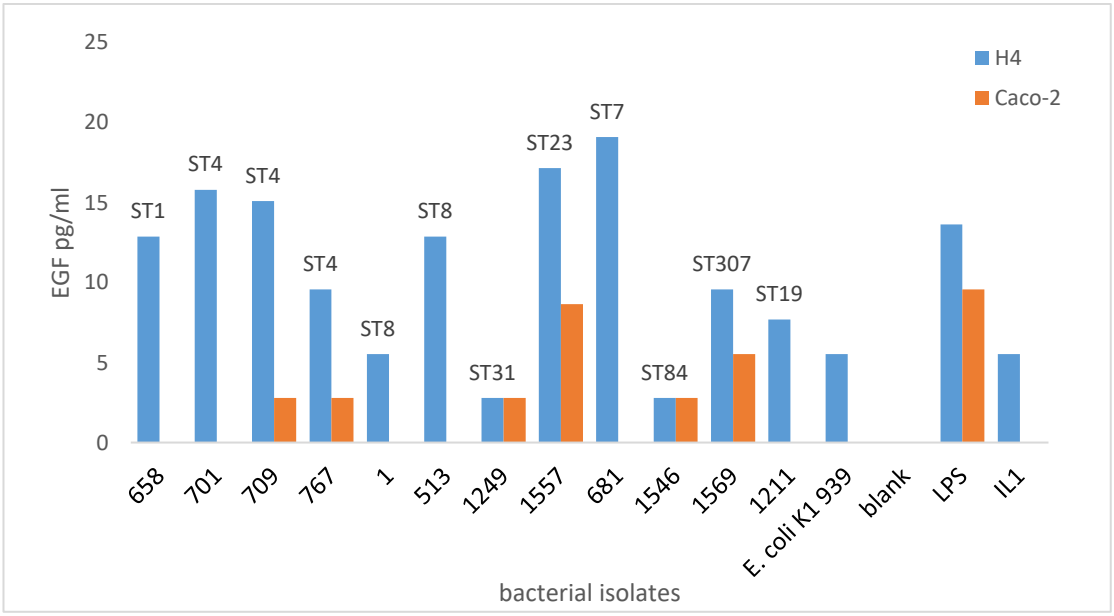


Figure 5-15. Bacteria induced EGF expression by human epithelial H4 and Caco-2 cells. Human cells were cultured in 24-well plates and infected with different bacterial isolates for 3 hours. After collection of cell supernatants, EGF levels were determined using Bio-Plex ELISA. . No statistical analysis between strains was performed for this assay as only one well was used and the result is an average of about 100 analyse of each well created by Luminex xMAB technology system, the instrument used in this assay at Nottingham City Hospital.

Epidermal growth factor (EGF) plays an important role in the intestinal development and endogenous repair following the gastrointestinal mucosa injury. Strains induced variable levels of stimulation in both cell lines ranging from lack of induction to 19 pg/ml in H4 cells in response to *C. malonaticus* ST7 strain 681 (Figure 5-15). All strains induced H4 cells to secrete EGF compare to 46% induced EGF in Caco-2 cells, and H4 was significantly higher than caco-2 cells (P<0.0001/ paired t test). The variation in the induction did not correlate with bacterial species or sequence types and no link was found between clinical history and level of induction. Both *C. sakazakii* ST8 strains had no effect on Caco-2 cells, which was also the case for IL-1β and MPC-1.

5.4 Activation of human Toll-Like Receptors by bacterial infection and genes expression.

Changes in gene expression underlie the alteration in cell physiology when in contact with bacteria. Gene expression in healthy tissue is different to that during inflammation and other illnesses. Quantitative polymerase chain reaction (qPCR) is one of the most accurate methods to investigate changes in gene expression. Here, qPCR was employed to study the effect of different bacterial strains on Human Toll-like receptors (TLRS) genes expression. Human TLRs family is one of the most important PRRs that are found on the cell membrane (Figure 5.16), and on the membranes of vesicle such as endosomes or lysosomes (Blasius and Beutler, 2010).

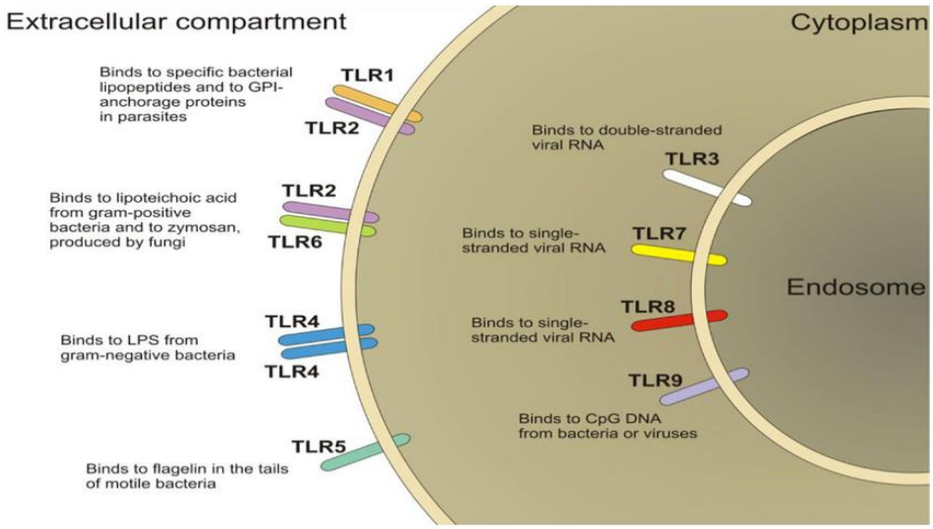


Figure 5-16. The site of expression of human Toll Like receptors (TLRs) and their ligands. TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 are found on the cell membrane whereas TLR3, TLR7, TLR8, and TLR9, are located in the endosomal membrane. Source: (Moloney *et al,* 2015).

H4 and Caco-2 cell lines were co-cultured with selected bacterial isolates from genus *Cronobacter* and one comparative strain from *E. coli* K1 for three hours. Then total RNA was extracted, and expression levels of TLRs were analysed by RT and qPCR. β–actin housekeeping gene was used as a reference gene, and untreated cells as a negative control in this study.

Expression of all investigated genes in absence of bacteria were compared to β-actin and GAPDH or only β-actin houskeeping genes. GAPDH and β-actin were normally detected in earlier cycles before most of other genes except TLRs 5-8 in H4 cells (Figure 5-17), whereas they were constantly expressed earlier in Caco-2 cells (Figure 5-18).

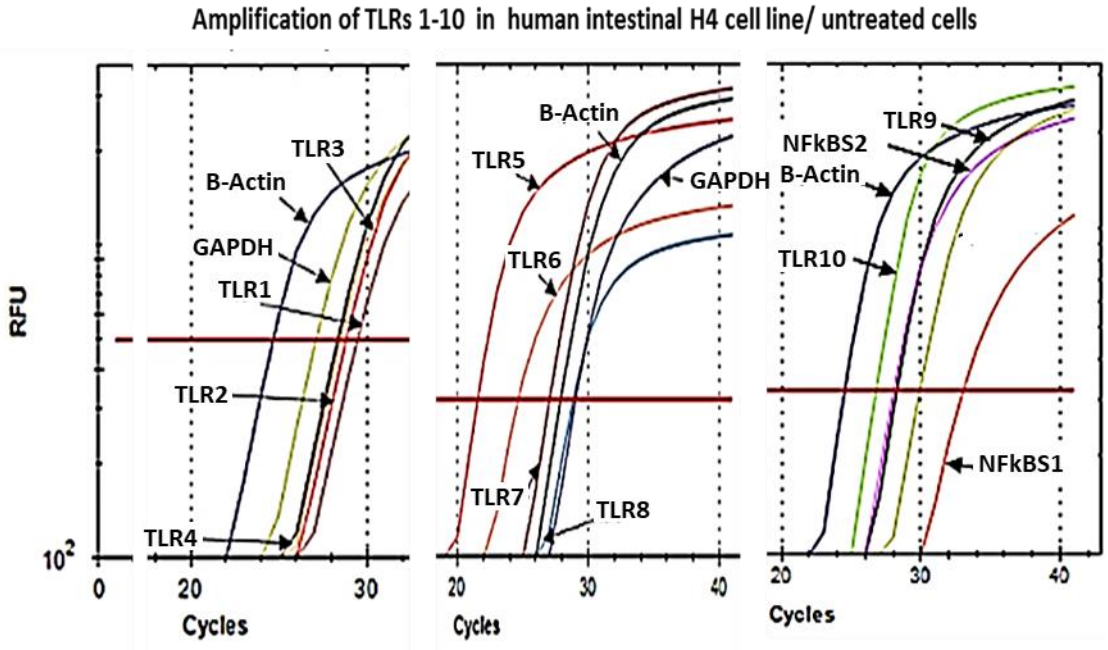


Figure 5-17 Expression of TLRs 1-10 in H4 cell line compared with housekeeping genes β–actin. RNA isolated from human cells, converted to cDNA and then gene expression analysed using q-PCR method. Primers of human TLRs obtained from InvivoGen, while NF-kB subunit 1 and 2 were designed in this study.

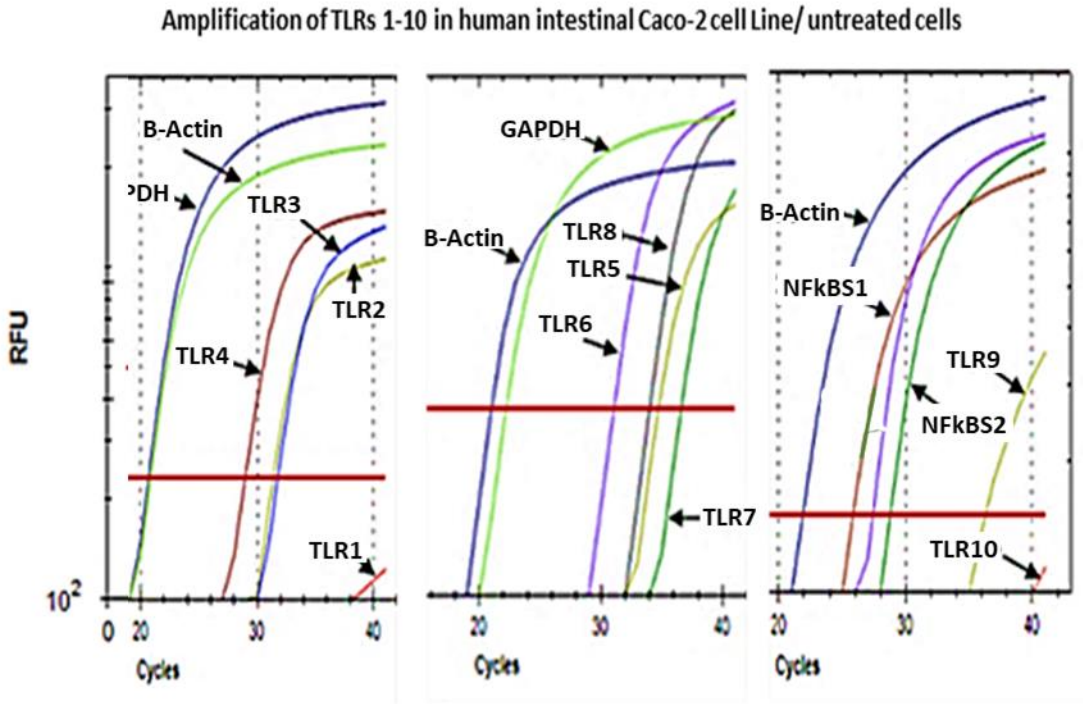


Figure 5-18 Expression of TLRs 1-10 in H4 cell line compared with housekeeping genes β–actin. RNA isolated from human cells, converted to cDNA and then gene expression analysed using q-PCR method. Primers of human TLRs obtained from InvivoGen, while NF-kB subunit 1 and 2 were designed in this study.

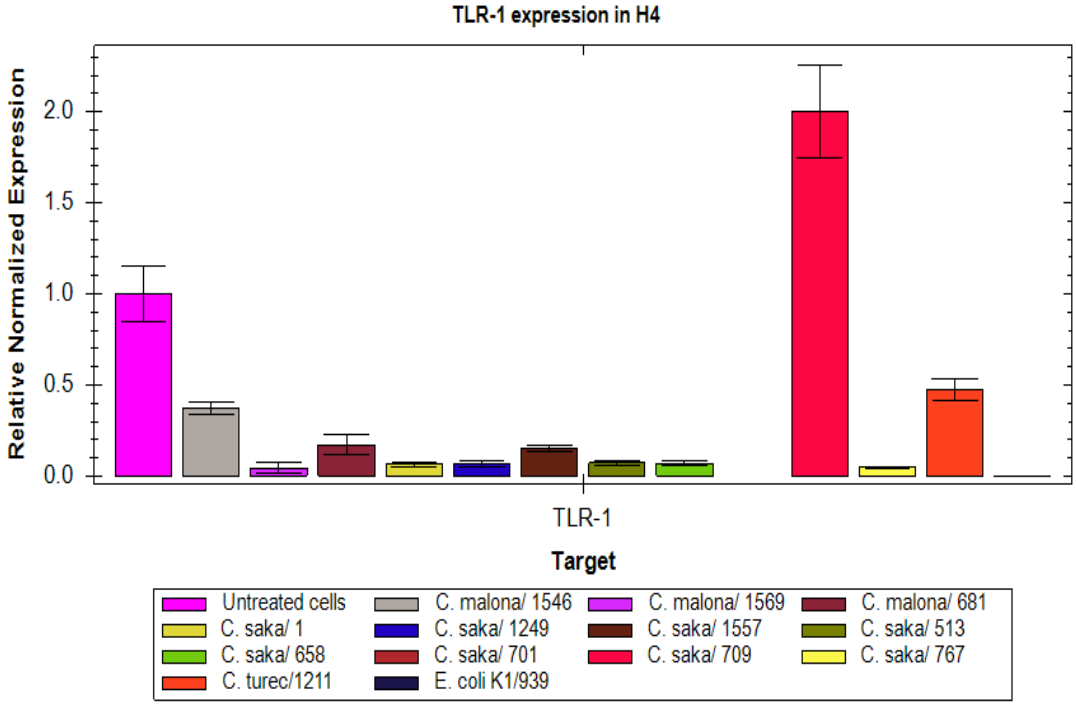


Figure 5-19 TLR1 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR1 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

However, no expression of TLR1 by Caco-2 cells was detected in response to all investigated stimulants, which might mean that this gene is not expressed in this cell line or it was affected by a mutation of the cells. In H4 cell line, only *C. sakazakii* strain 709 upregulated the expression of TLR1 in H4 cells 3 hours after incubation, compared with other strains. Most of bacterial isolates downregulated TLR1 expression by up to 90% in H4 cells compared with untreated cells. Incubation of H4 cells with *C. sakazakii* ST4 strain 701 resulted in silencing of the gene expression of TLR1 (Figure 5-20). *E. coli* K1 suppressed TLR1 expression 50 to 2540 folds more than *Cronobacter* isolates with the exception of *C. sakazakii* ST4 strain 701 which also completely blockedthe expression of this gene.

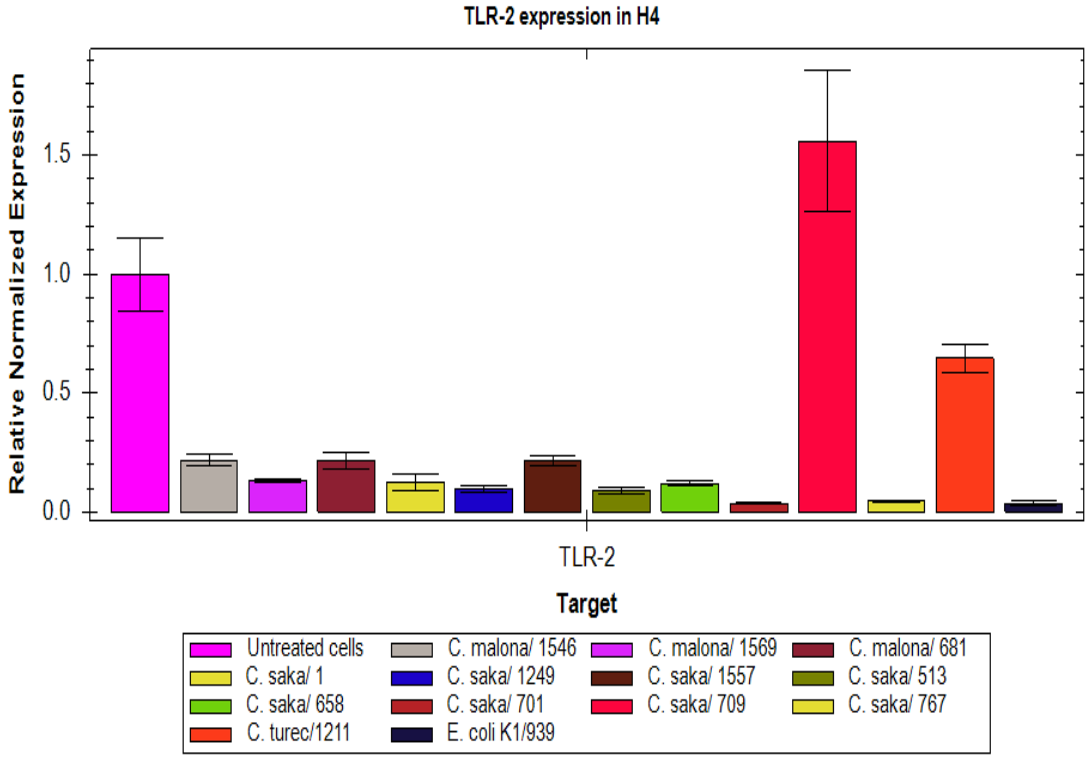


Figure 5-20 TLR2 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

Similar results were obtained by TLR2. H4 cells showed decreased expression of this TLR in response to 93% of bacterial isolates (Figure 5-20). *C. malonaticus* strains downregulated the expression by 75% of control, while *C. sakazakii* ST4 strains 701 and

767, and *E. coli* K1 suppressed the expression by up to 90%. Interestingly, only *C. sakazakii* ST4 strain 709 upregulated the gene expression by about two-fold. This strain is associated with neonatal septicaemia in French outbreak 1994. *C. turicensis* showed a minor effect, and reduced the expression by approximately 25%. *E. coli* K1 reduced the expression of TLR2 by up to 57-fold compared to the *Cronobacter* isolates.

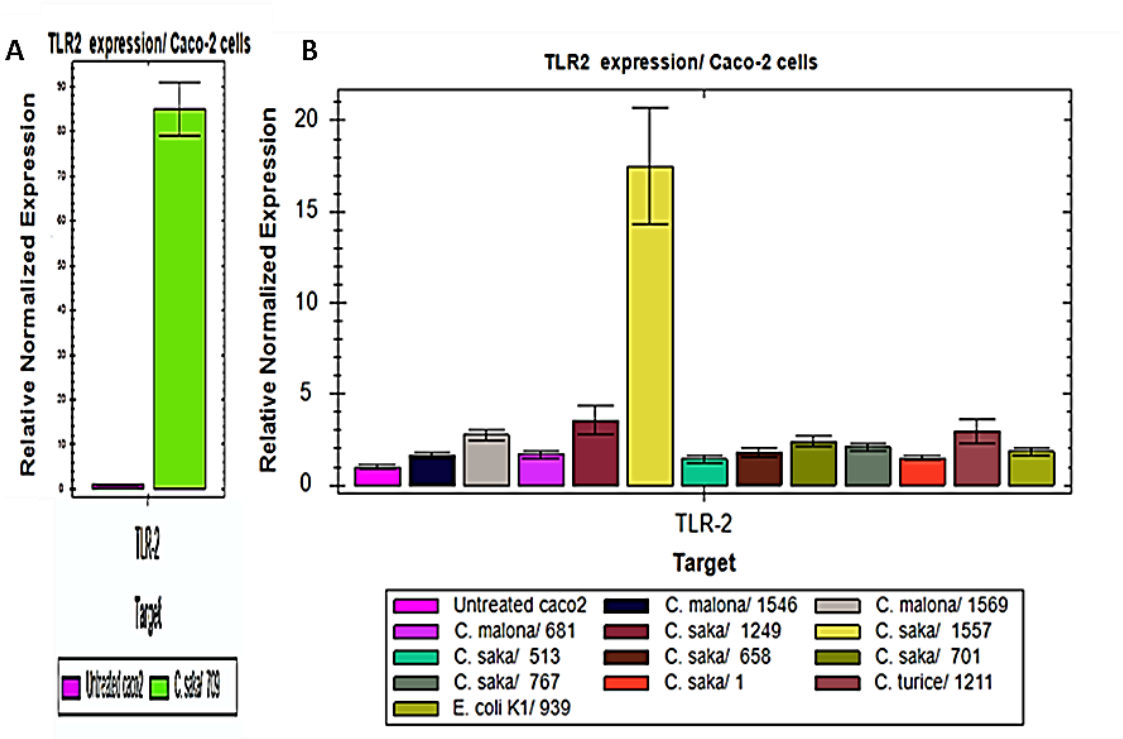


Figure 5-21 TLR2 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* was highly upregulated the TLR2 expression in Caco-2 cells compared to untreated cells. **B)** Strainses that showed 20-fold or less

In contrast, incubation of these bacterial isolates with Caco-2 cells resulted in upregulated TLR2 expression. Most bacterial strains increased the expression by an average of 2-5-fold. *C. sakazakii* ST23 strain 1557 enhanced the expression of this gene about 18-fold compared to the blank where no bacteria had been added to the cells. *C. sakazakii* ST4 strain 709 produced the highest induction of gene expression, which was more than 100-fold higher than for untreated cells (figure 5-21). *C. sakazakii* ST8 strains 1 and 513 displayed the lowest activation among all of investigated strains. However,

these two isolates were among the lowest cytokines inducers of all investigated strains. *E. coli* K1 was included in this project as a comparative strain from a different genus. This strain showed high similarity in terms of activating this receptor and showed about 2-fold increase in the expression.

Most of the bacterial isolates suppressed the expression of TLR3 ranging from 60% to completely undetectable, with exception of *C. turicensis* strain 1211, which showed only about 20% reduction in the TLR3 expression. *C. malonaticus* strains 1546 and 681 inhibited expression of this gene by about 60% while the reduction observed by strain 1569 was about 80% from the gene expression in untreated cells. Some bacterial isolates such as *C. sakazakii* 1557 and *E. coli* K2 inhibited TLR2 by more than 90% (Figure 5-22). Similarly, *E. coli* showed more ability to downregulate human TLRs than *Cronobacter* spp. isolates. However, *C. sakazakii* strain 709 again showed highest level of gene expression for TLR3.

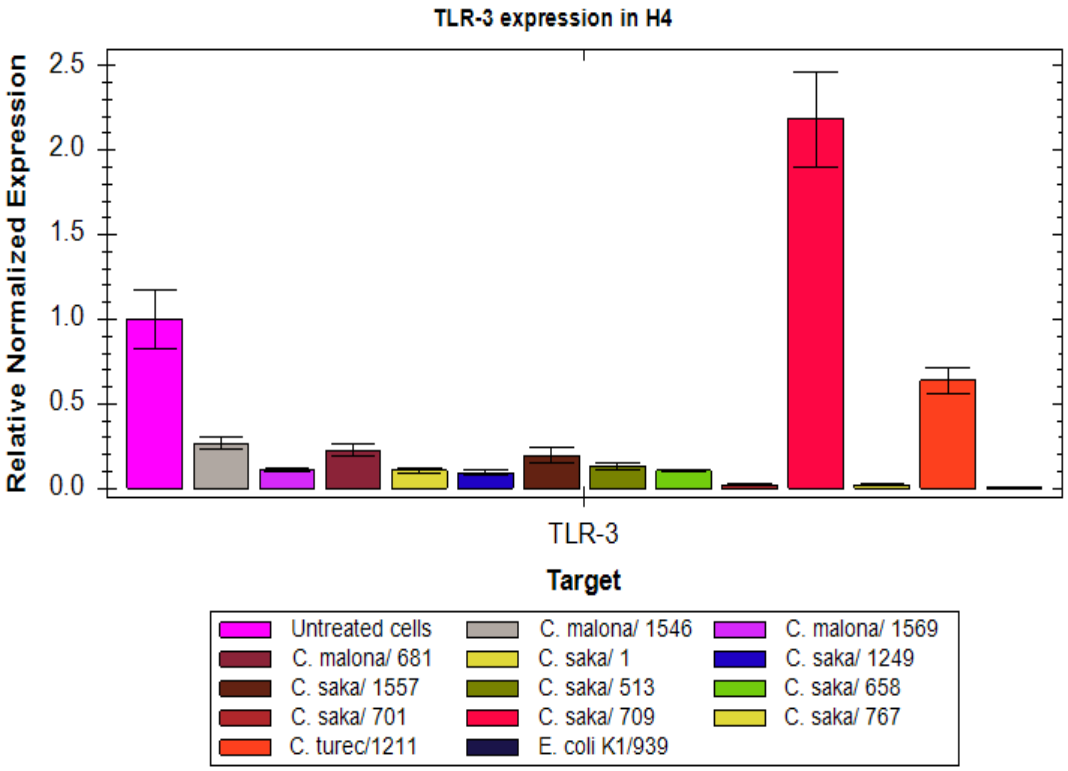


Figure 5-22 TLR3 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

Caco-2 cells revealed an increase in TLR3 expression in response to about 85% of bacterial isolates. Very high increase was recorded by Caco-2 cells in response to *C. sakazakii* ST4 strain 709, which is about 150-fold higher than untreated cells followed by *C. sakazakii* ST23 strain 1557 which induced this gene by about 25-fold of control where no bacteria were added to the cells (Figure 5-23-A). *C. malonaticus* strain 1546 and *C. sakazakii* strain 1 showed no effect on the gene expression (Figure5-23-B). Minor increase observed in response to to *C. sakazakii* strains 1249 and 513, while the increase in the expression in response to the rest of the strains ranged from 1.5 to 2.1 folds of untreated cells. These results are largely different to those recorded by H4 cells where the most strains suppressed the expression and only *C. sakazakii* ST4 strain 709 showed a two-folds increase.

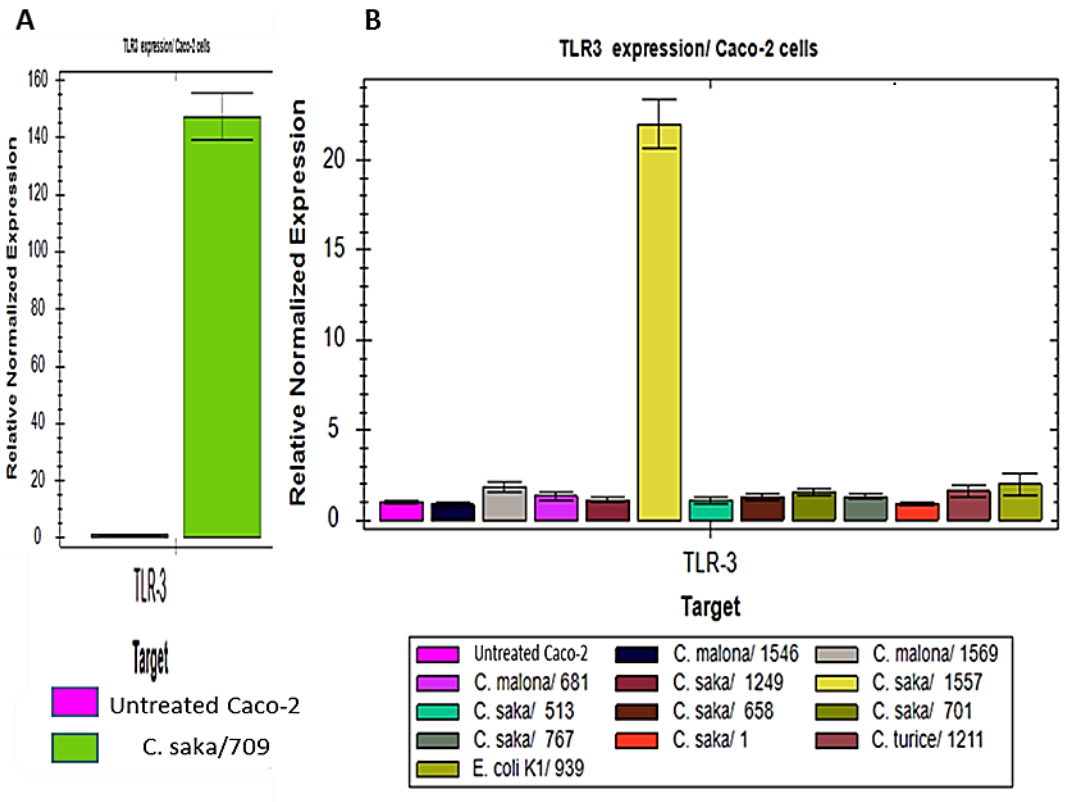


Figure 5-23 TLR3 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* was highly upregulated the TLR3 expression in Caco-2 cells compared to untreated cells. **B)** Strainses that showed 25-fold or less

Likewise, the expression of human TLR4 in H4 cells was suppressed by most of bacterial isolates (Figure 5-24). The inhibition ranged from 10% showed by *C. turicensis* strain 1211 to about 100%, recorded for *C. sakazakii* ST4 strains 701 and 767 and *E. coli* K1 strain 939. *C. malonaticus* strain 1569 and *C. sakazakii* strains 1, 1249 and 658 inhibited 80% of the level of gene expression in untreated cells. *C. malonaticus* strains 1546 and 681 and *C. sakazakii* strain 1557 inhibited the expression by about 70%. Only *C. sakazakii* ST4 strain 709 again upregulated this gene by about 1.7-fold compared to untreated cells.

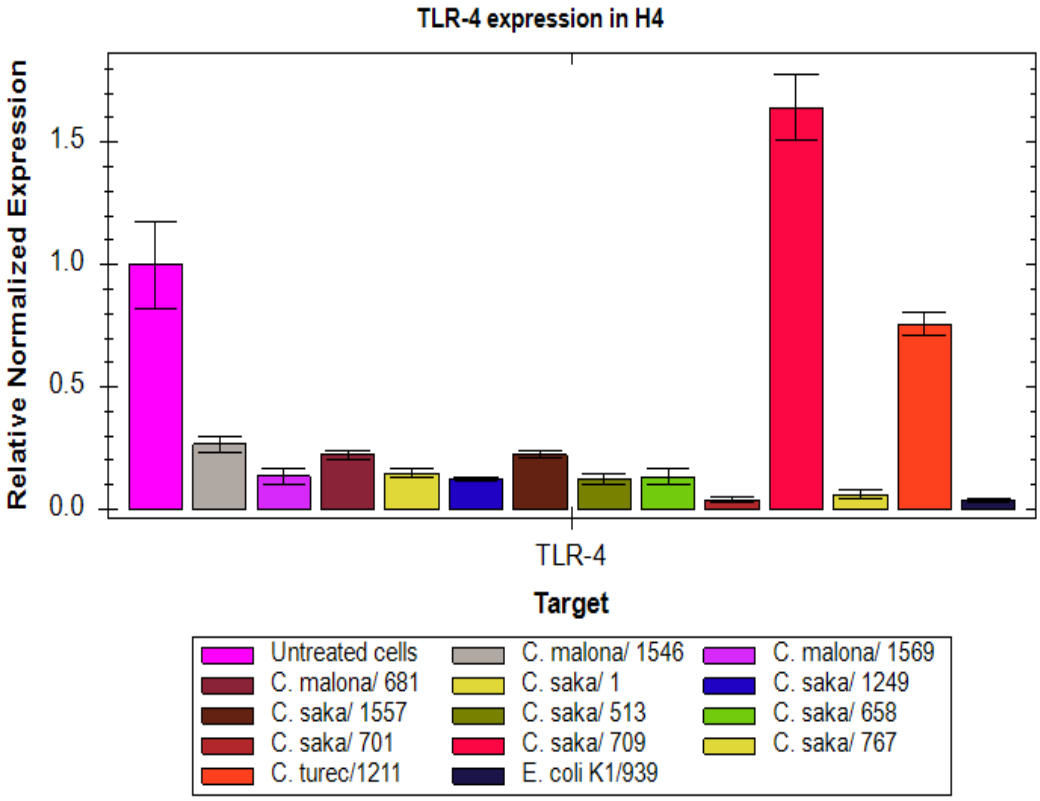


Figure 5-24 TLR4 expression H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

With regard to Caco-2 cells, TLR4 was upregulated by all bacterial strains. The highest recorded expression was in response to *C. sakazakii* ST4 strain 709 which is about 45-fold higher compared to untreated cells (Figure 5-25-A).

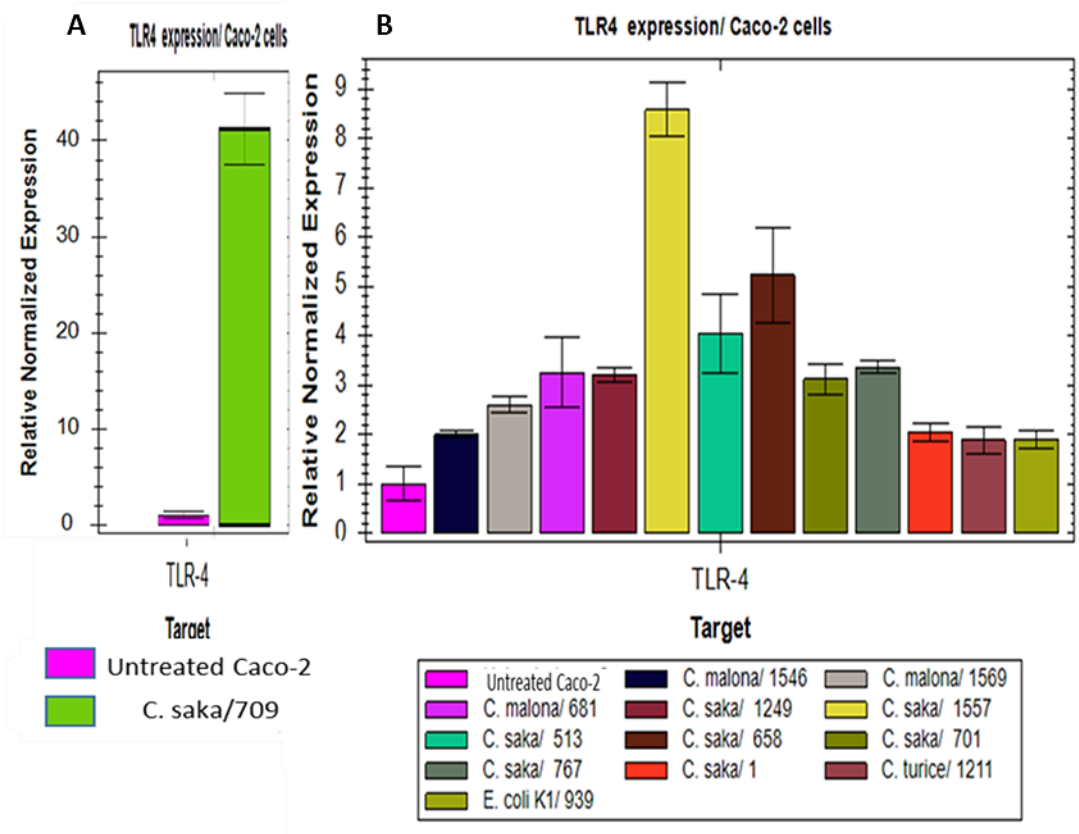


Figure 5-25. TLR4 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. asakazakii* 709 was highly upregulated the TLR3 expression in Caco-2 compared to untreated cells. **B)** Strainses that showed 9-fold or less.

C. sakazakii ST23 strain 1557 induced an increase of the expression of about 9-fold (Figure 5-25-B), followed by *C, sakazakii* ST1 strain 658 that induced the expression about 5 times higher than the untreated cells. Fourfold increases were observed by *C. malonaticus* strain 1569, *C. sakazakii* strain 513 and *C sakazakii* strain 767, while the enhancement of the gene expression by other strains ranged from 2-3 fold.

The expression of TLR5 in H4 cells was upregulated by some strains and downregulated by others, while no effect was observed by *C. sakazakii* strain 767. *C. sakazakii* strain 1557 enhanced the gene expression about 20-fold higher than in the non-treated cells (Figure 5-26-A), while *C. sakazakii* strain 1249 and *C. turicensis* increased the expression by 7 and 6-fold respectively. *C. malonaticus* strain 681 showed minor inhibition (Figure 5-26- B).

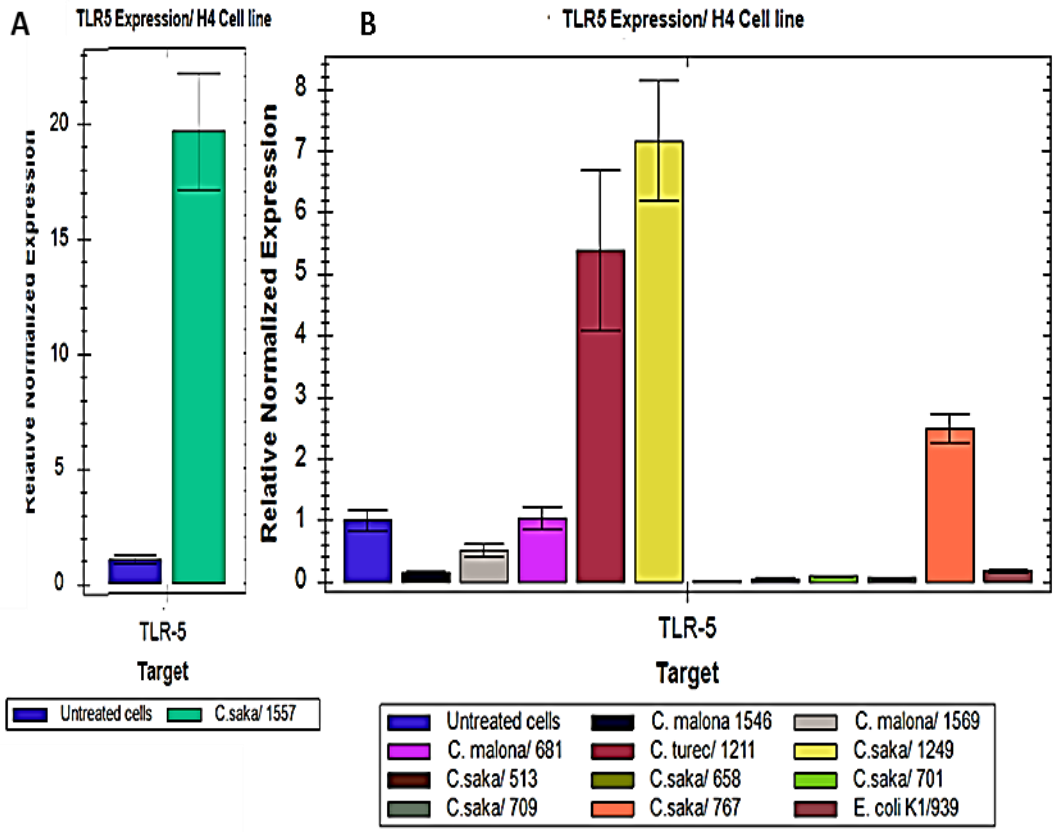


Figure 5-26 TLR5 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR5 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* 1557 was highly upregulated the TLR5 expression in H4 cells compared to untreated cells. **B)** Strainses that showed 8-fold or less.

C. sakazakii ST8 strains showed about 100% inhibition. Unexpectedly, *C. sakazakii* strain 709 almost completely blocked the expression of this gene, which is completely different to the results obtained from the other TLRs.

Conversely, TLR5 in Caco-2 cells was more inducible by bacterial infection. *C. sakazakii* ST4 strain 709 enhanced the expression up to 2500 times higher than the non-stimulated cells, whereas it was almost totally blocked by this strain in H4 cells. In addition, *C. sakazakii* ST23 strain 1557 displayed the ability to upregulate this gene more than 450-fold higher than in untreated cells that used as negative control in this assay (Figure 5-27-A). *E. coli* K1 caused the third highest upregulation of this gene in |Caco-2 cells (Figure 5-27-B), and increased the expression by about 25 times, whereas *C. malonaticus* enhanced the expression by about 17-fold compared to non-treated cells.

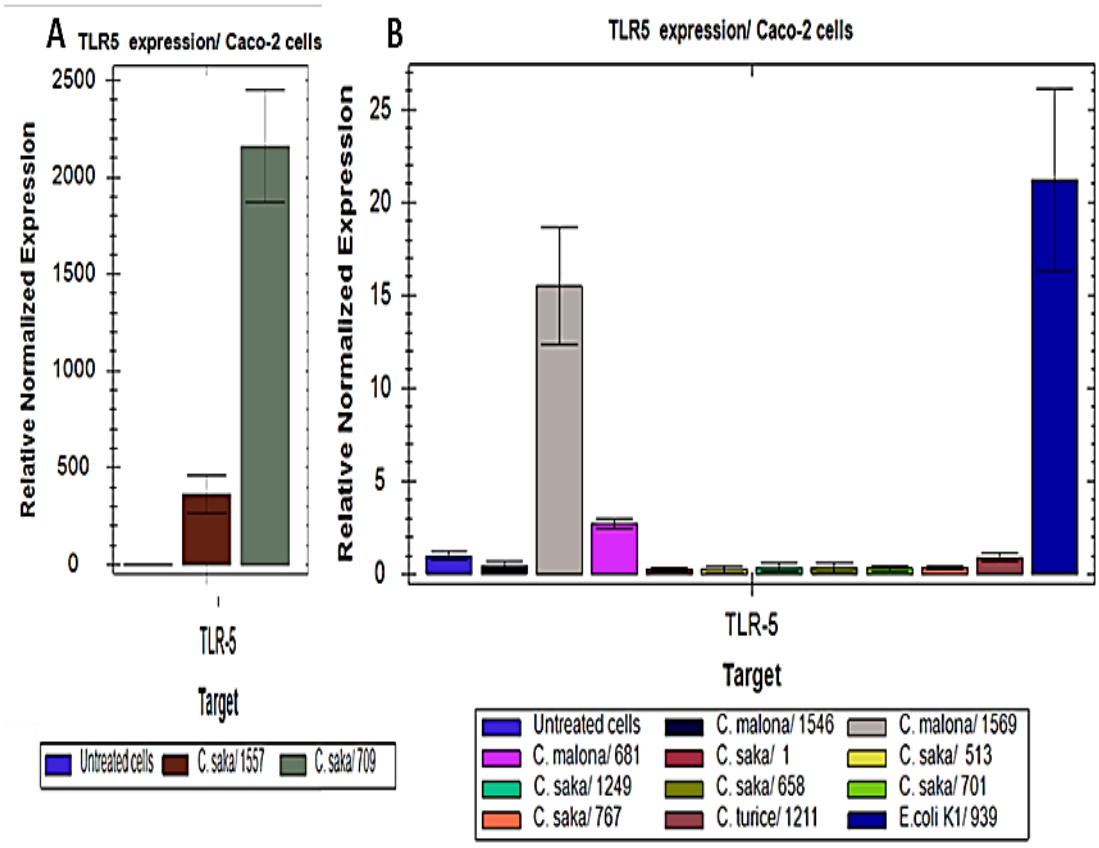


Figure 5-27 TLR5 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 and 1557 highly upregulated the TLR5 expression in Caco-2 (450 and 2500-fold respectively) compared to untreated cells. **B)** Strainses that showed 20-fold or less.

About a threefold increase was caused by *C. malonaticus* strain 681, while the rest of the strains downregulated this gene. These results show differences between the response of adult and neonatal cell lines receptors to the same pathogens. Upregulation of TLR5 in Caco-2 cells by some strains could be an important factor of the controlled immune response and haemostasis generated by adult epithelial cells toward bacterial interaction.

The effect of cultured bacteria with H4 cells on TLR6 expression was varied. Some strains reduced the expression of this gene by up to 90%, while others upregulated its expression. *C. turicensis* 1211 and *C. sakazakii* 1557 and 1249, upregulated this gene by 160, and 80 and 50-fold respectively (Figure 5-28-A), while *C. sakazakii* strain767 increased the expression of this gene 30-fold higher than in the untreated cells (Figure

5-28-B). Interestingly, *C. sakazakii* ST4 strain 70 9 did not affect the expression of this gene. *C. malonaticus* strains showed variable effects on this TLR. Strains 681 and 1569 increased the expression by about 2 and 10-fold respectively, while strain 1546 downregulated this gene by about 50%. Among *Cronobacter spp.* isolates, strain 658 was the most suppressive strain and blocked about 99% of expression of this gene. However, *E. coli* K1 also did not affect the expression of this gene.

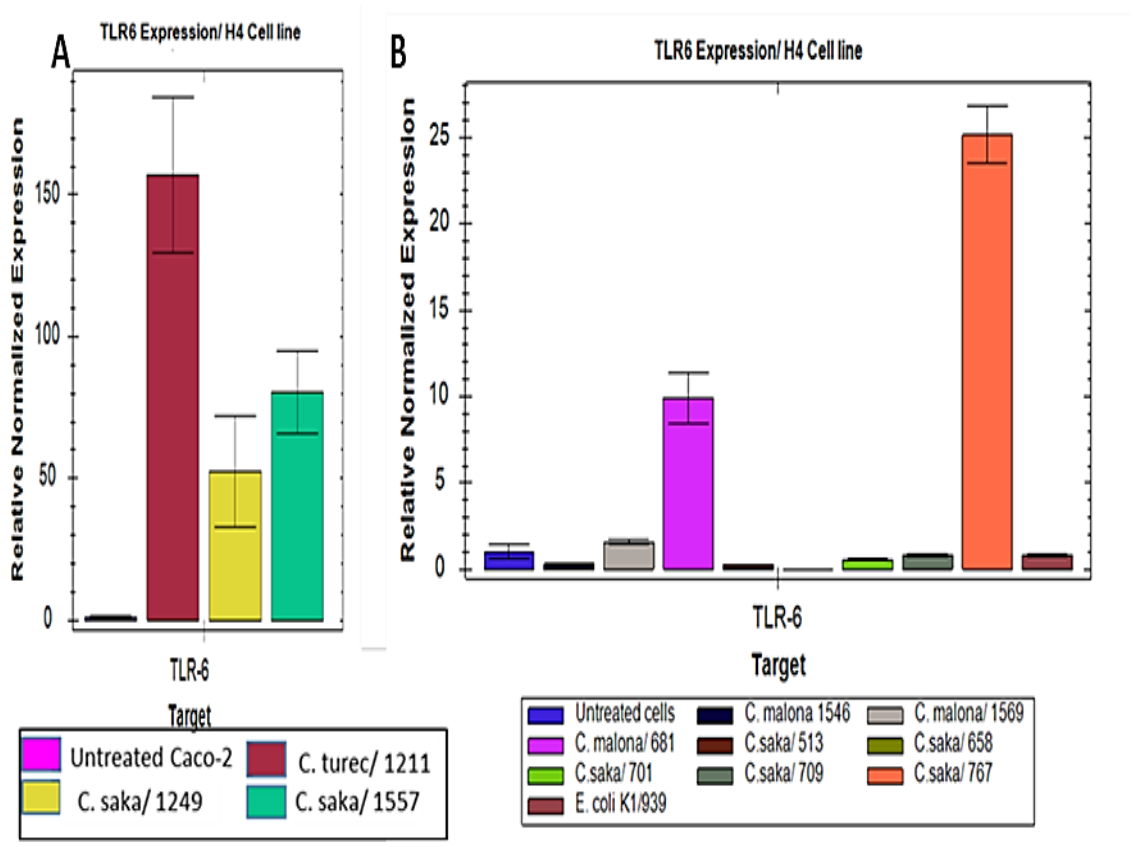


Figure 5-28 TLR6 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 1249 and 1557 and *C. turicensis* strain 1211 that highly upregulated the TLR6 expression in H4 (80-150-fold) compared to untreated cells. **B)** Strainses that showed 25-fold or less.

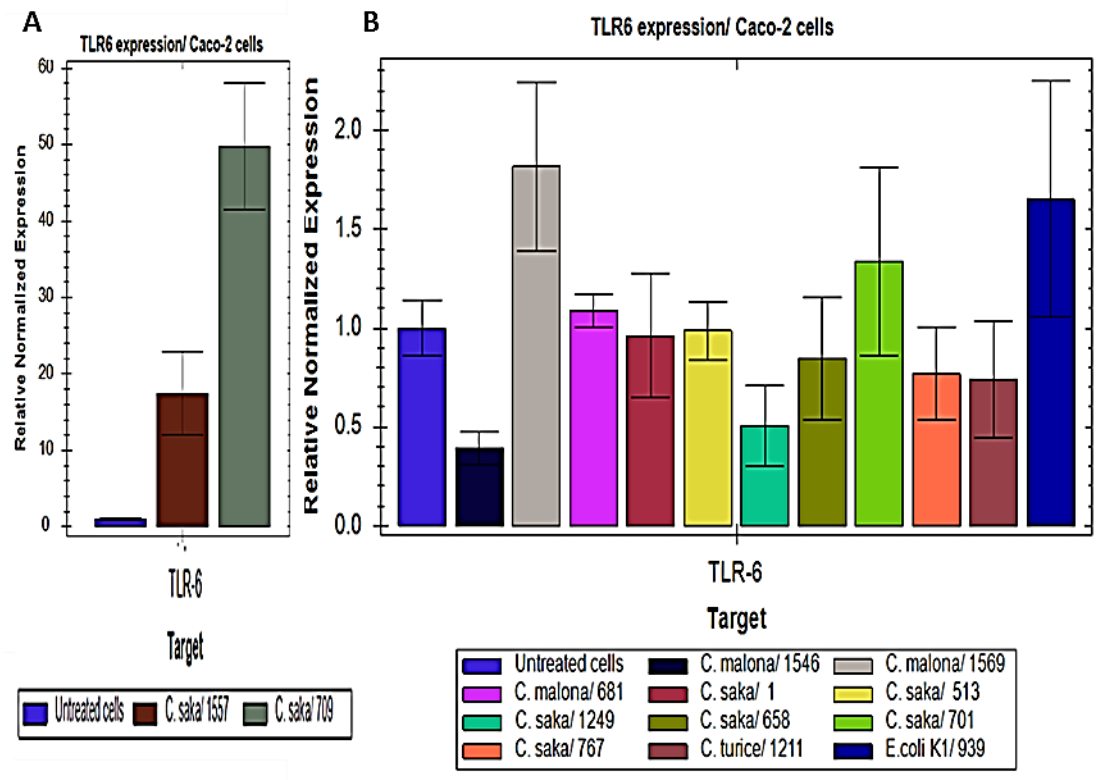


Figure 5-29. TLR6 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 and 1557 highly upregulated the TLR6 expression in Caco-2 cells (18-50-fold) compared to untreated cells. **B)** Strains that showed 2-fold or less.

With regard to Caco-2 cells, only *C. sakazakii* strains 709 and 1557 displayed capability to upregulate TLR6 to a high degree and recorded approximately 50 and 20-fold higher expression than in untreated cells respectively (Figure 5-29-A). *C. malonaticus* strain 1569 showed only two fold enhancement, while *E. coli* K1 increased the expression by about 1.5-fold. A minor increase was induced by *C. malonaticus* strain 681 and *C. sakazakii* 701, while strains *C. malonaticus* strain 1456 and *C. sakazakii* 1249 downregulated the expression by about 60% and 50% respectively (Figure 5-29-B). *C. sakazakii* ST8 strain 1 and 8 did not effect this gene and *C. sakazakii* strains 658 and 767 and *C. turicensis* strain 1211 showed minor reduction in the expression of TLR6 in this cell line.

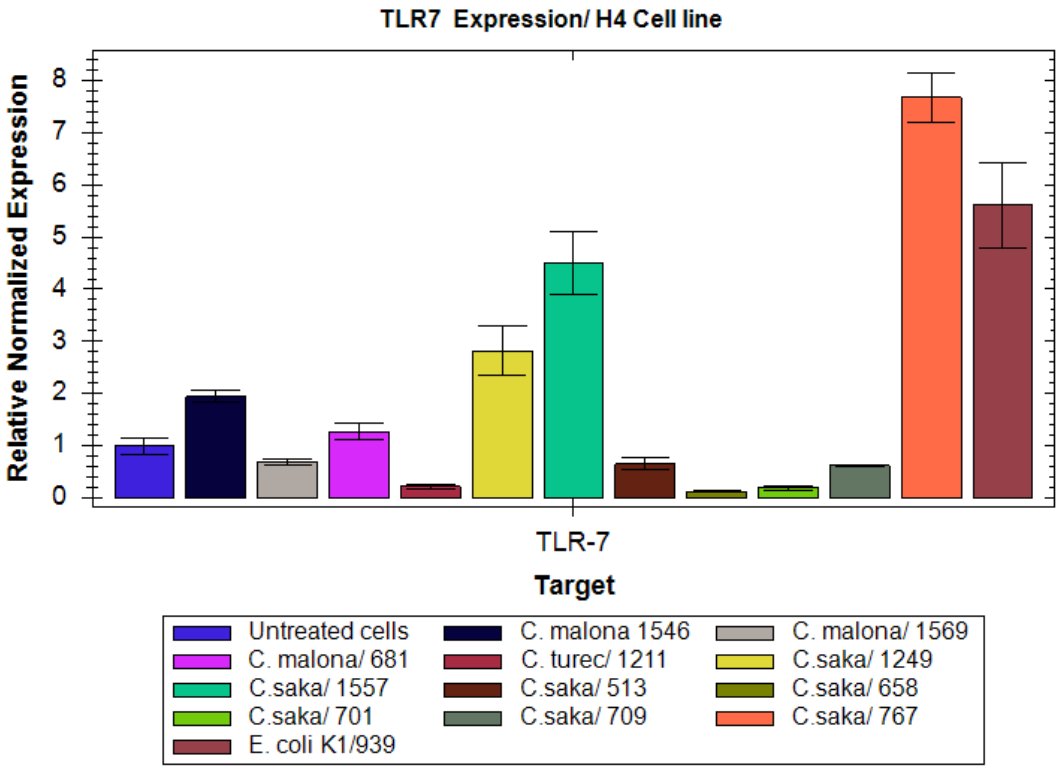


Figure 5-30. TLR7 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and by RT and qPCR were used to analyze the expression levels of TLR7. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The pattern of TLR7 expression in H4 cells in response to the investigated bacterial isolates was varied. The gene was highly downregulated by *C. turicensis* strain 1211 and *C. sakazakii* strains 658 and 701 by more than 90%. Slight suppression of TLR7 was recorded by *C. malonaticus* strain 1569 and *C. sakazakii* strains 513 and 709. However, a minor increase in the gene expression was obtained by *C. malonaticus* 681, while a two-fold increase was seen when cells were incubated with strain 1546. *C. sakazakii* ST4 strain 767 which is associated with fatal meningitis in French 1994 outbreak showed 8-fold increase in TLR7 expression compared with strains from same ST group and also linked to fatal infection such as NECIII (strain 701) (Figure 5-30). *C. sakazakii* strains 1249 and 1557 caused 3 and 4 fold increases in TLR7 expression in H4 cells while *E. coli* caused about a 6-fold increase.

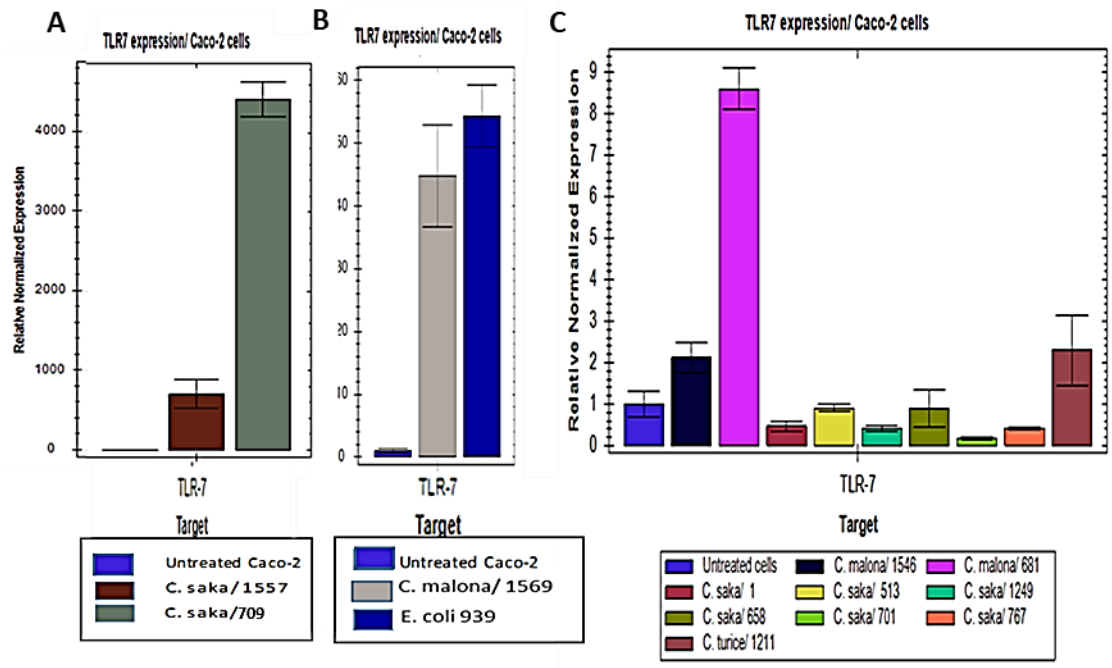


Figure 5-31. TLR7 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 and 1557 were highly upregulated the TLR7 expression in Caco-2 cells (800-4500-fold) compared to untreated cells. **B)** C. malonaticus and E. coli K1 that showed 45 and 55-fold higher than untreated cells. **C)** Strains that inceased the expression by 9-fold or less.

The expression of TLR7 in Caco-2 cells was markedly increased by some strains. *C. sakazakii* ST4 strain 709 upregulated the expression by more than 4500-fold , and about 800-fold was also recorded *C. sakazakii* ST23 strain 1557 in Caco-2 cells compared to non-treated cells (Figure 5-31-A). Culturing with *C. malonaticus* strain 1569 and *E. coli* K1 strain 939 upregulated TLR7 expression 54 and 44-fold of untreated cells respectively (Figure 5-31-B). A twofold increase was obtained when cells were incubated with *C. turicensis* strain 1211. *C. sakazakii* ST4 strains 701 and 767 downregulated the gene by 80% and 60% respectively which is different to the results obtained for strain 709. *C. sakazakii* ST8 strains 1 and 513 suppressed transcription of this gene by 50% and 10% respectively (Figure 5-31-C).

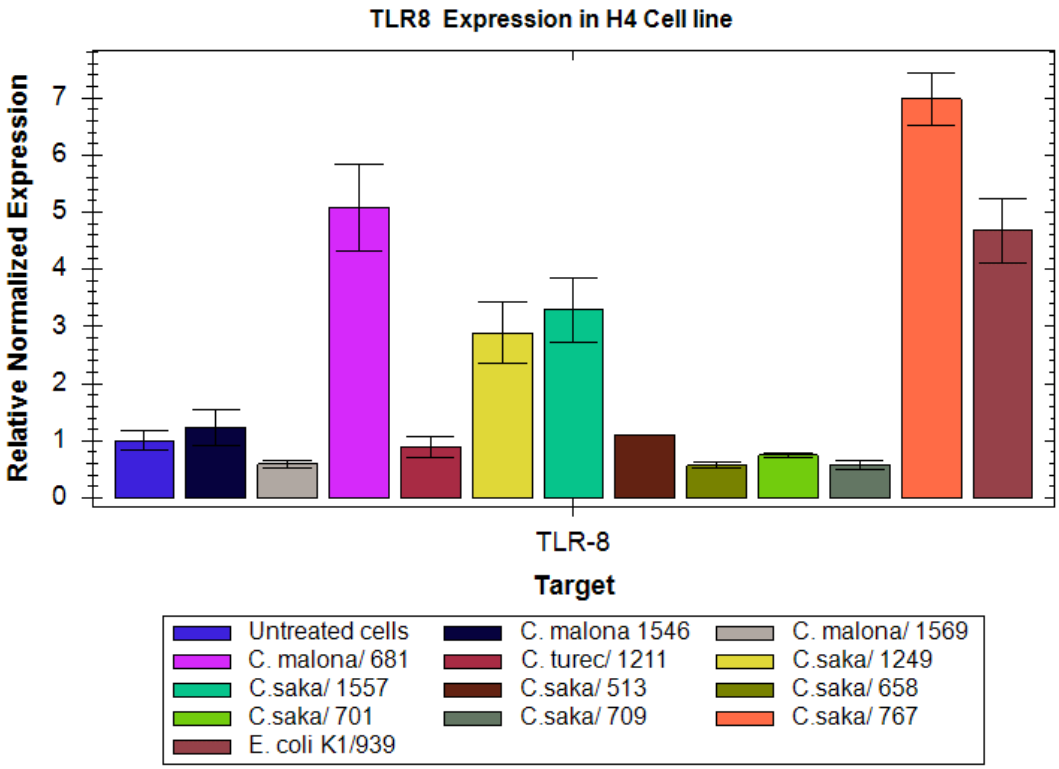


Figure 5-32. TLR8 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The expression of TLR8 by H4 cells due to incubation with bacteria was variable. *C. malonaticus* strains showed varied effects on this cell line. Strain 1546 slightly upregulated gene transcription, while strain 1569, which is responsible for neonatal severe meningitis downregulated about 40% of gene expression in untreated cells. However, *C. malonaticus* strain 681, the type species in NTU collection, upregulated this gene by about 5-fold compare to the non-treated cells. With regard to *C. sakazakii* strains, the results were also variable and no correlation was observed between the sequence type and effect on the cell line. Treating with strains 1249 and 1557 resulted in gene upregulation by 3 and 3.5-fold of the untreated cells, while strains 658, 701 and 709 downregulated the expression (Figure 5-32). *C. sakazakii* ST8 strain 513 showed a minor upregulation of TLR8 in H4 cells while no detectable expression was obtained when cells were treated with strain 1 from the same ST. The highest increase of gene expression was recorded by *C. sakazakii* ST4 strain 767 that was associated with neonatal fatal meningitis. Only 10% downregulation was recorded when H4 cells were

co-cultured with *C. turicensis.* Comparatively, *E. coli* K1 strain upregulated this gene approximately fivefold.

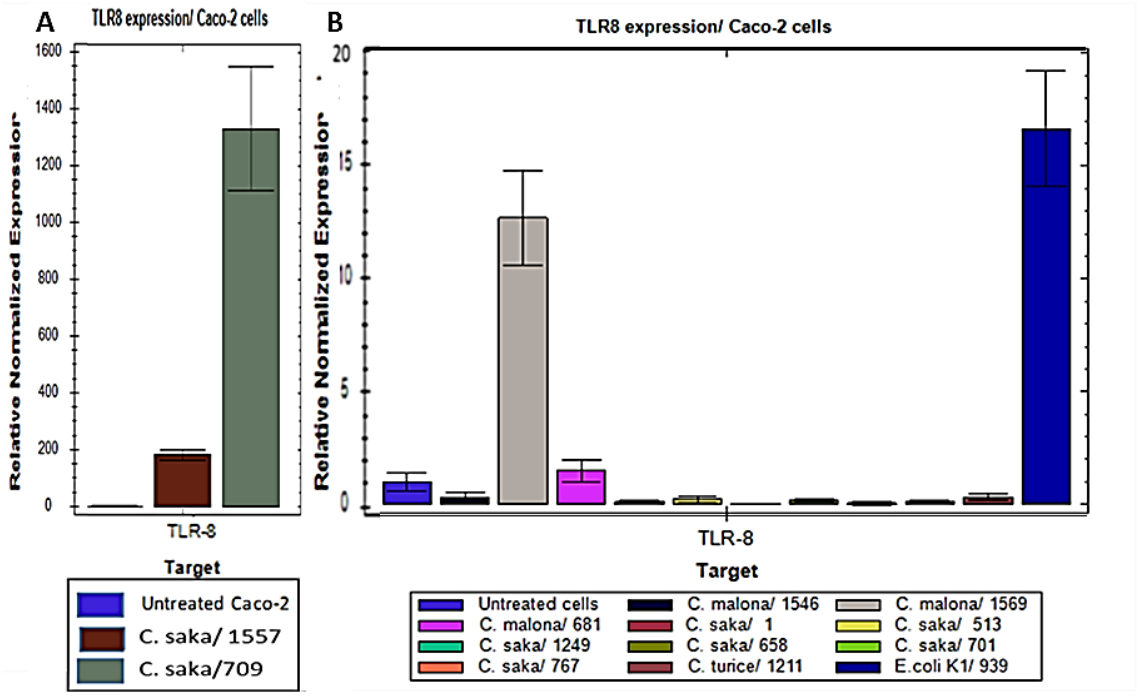


Figure 5-33. TLR8 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 and 1557 were highly upregulated the TLR8 expression in Caco-2 cells (200-1330-fold) compared to untreated cells. **B)** Strains that inceased the expression by 12-fold or less.

Caco-2 cell line showed a different response in terms of activation of TLR8 by co-culturing with selected bacterial isolates. *C. sakazakii* ST4 strain 709 exhibited the highest upregulation of this gene in Caco-2 cells and showed about a 1330-fold increase in expression of this gene compared to untreated cells followed by about 200-fold increase obtained when cells were treated with strain 1557 (Figure 5-33-A). 61% of bacterial isolates negatively affected the gene and downregulated its expression by up to 99% including all of the remaining *C. sakazakii* strains (Figure 5-33-B). The meningitic *C. malonaticus* strain 1569 upregulated this gene 13-times higher than in untreated cells, while only a 1.5 increase was obtained by strain 681. *E. coli* K1 increased TLR8 expression in Caco-2 cells by about 17-fold.

The effect of culturing of H4 cells with the investigated bacterial isolates on the expression of TLR9 was varied. Only *C. sakazakii* ST4 strain 709 that is associated with neonatal septicaemia was able to upregulate the expression of this gene. No effect of treating with *C. turicensis* was seen on the gene transcription. Other strains downregulated this gene, and cleare suppression was obtained by *C. sakazakii* 701 and *E. coli* K1 that suppressed expression of this gene bymore than 99.9%.

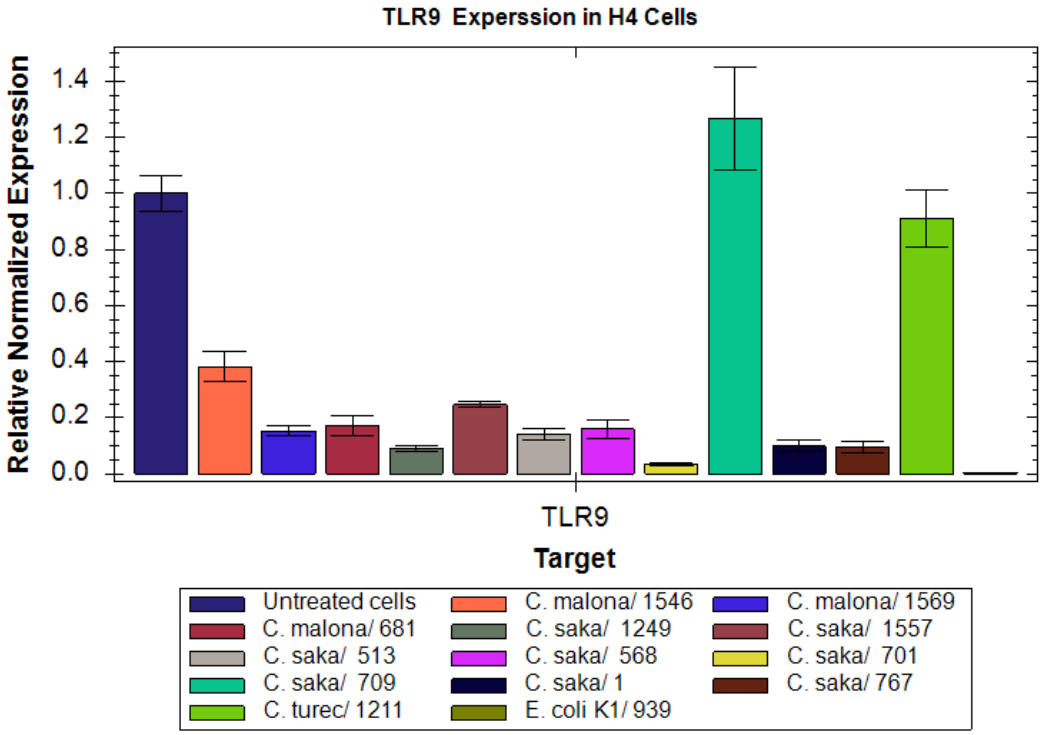


Figure 5-34. TLR9 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The suppression caused by other strains ranged from 70% to 90% and the latter was recorded by bacterial strains associated with serious illnesses such as meningitis which showed for strain 1249 and 767 (Figure 5-34).

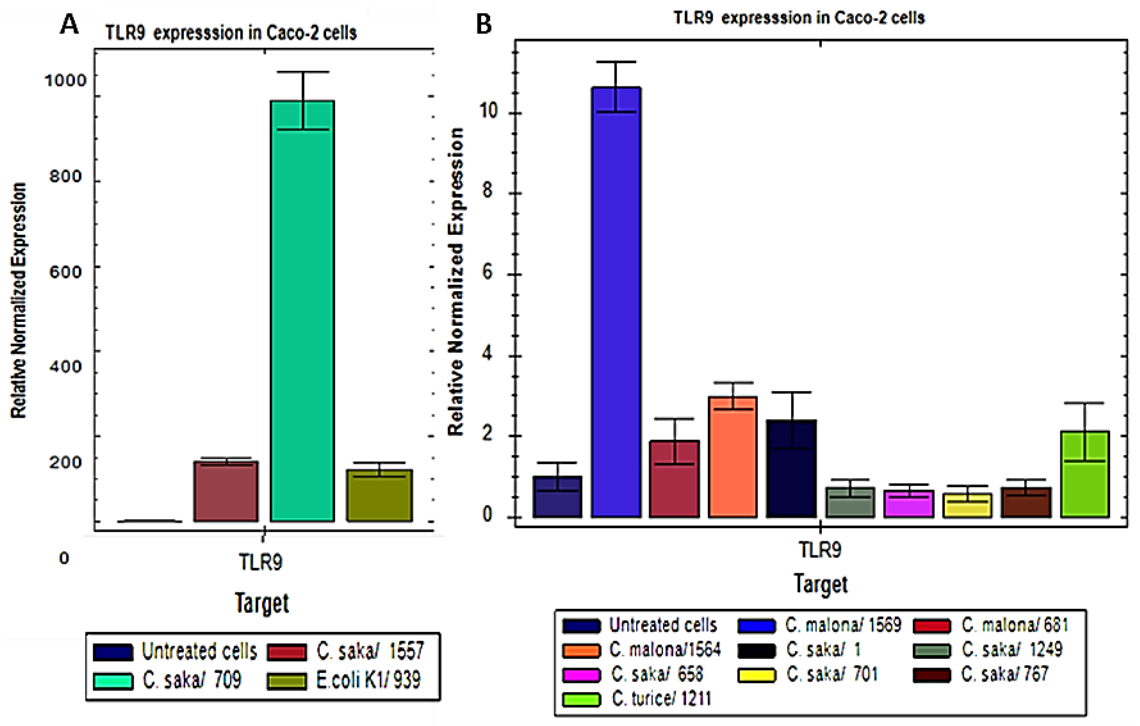


Figure 5-35 TLR9 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 1557 and 709, and *E. coli* K1 that were highly upregulated the TLR8 expression in Caco-2 cells (200-950-fold) compared to untreated cells. **B)** Strains that inceased the expression by 12-fold or less.

With regard to Caco-2 cells, TLR9 was affected differently compared to H4 cells. This gene was upregulated significantly by *C. sakzakii* 709 (978-fold), *E. coli* K1 (178-fold) and *C. sakzakii* 1557 (138-fold) (Figure 5-35-A). However, among the other *C. sakazakii* strains, only strain 1 showed about a two-fold increase in gene expression, while strains 1249, 658, 701 and 767 dowenregulated this gene. All of *C. malonaticus* upregulated the expression of TLR9 in Caco-2 with values ranging from 2-9 fold, and a twofold increase was seen when cells were treated with *C. turicensis* strain 1211(Figure 5-35-B).

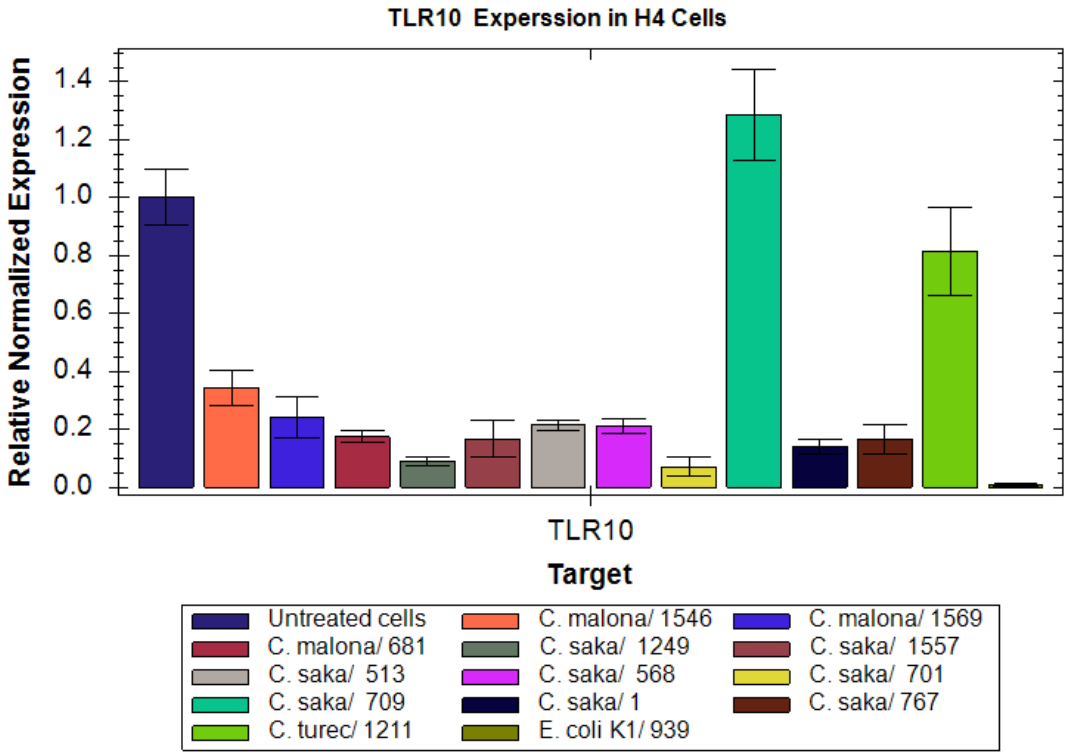


Figure 5-36. TLR10 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The expression of TLR10 in H4 cells was downregulated by 92% of bacterial isolates (12/13) and one *C. sakazakii* ST4 strain 709 exhibited a minor upregulation to about 110% of control. The reduction of the gene expression ranged from 30% for *C. turicensis* strain 1211 to approaching 100% for *E. coli* K1. *C. malonaticus* strains suppresed this gene by a range of 75% to 85%, while reduction this gene expression in response to *C. sakazakii* strains ranged from 83% to 99% of the expression in untreated cells.

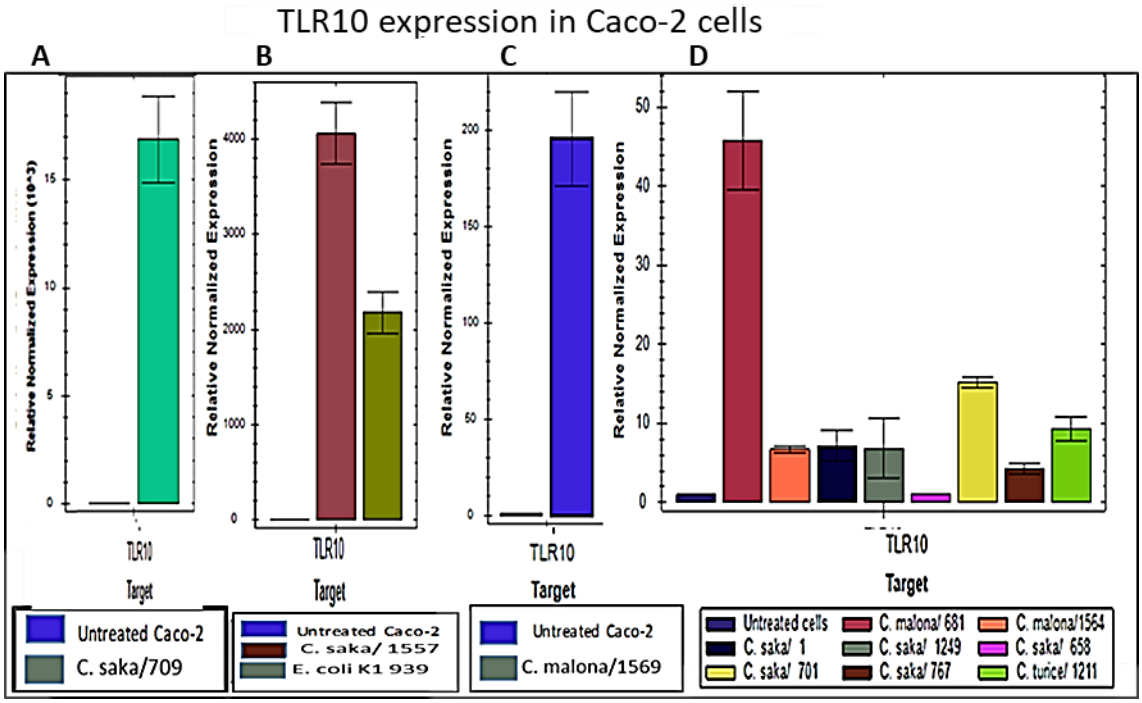


Figure 5-37 TLR10 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 that highly upregulated the TLR10 expression in Caco-2 cells (about15500-fold). **B)** *C. sakazakii* 1557 and *E. coli* that highly upregulated the TLR10 expression by (2200 and 4100-fold respectively compared to untreated cells. **C)** *C. malonaticus* 1559 that showed about 200-fold higher than untreated cells. **D)** Strains that inceased the expression by 50-fold or less.

Treating Caco-2 cells with the selected bacterial strains resulted in upregulation of TLR10 by all strais. The lowest increase was about 106% recorded for *C. sakazakii* strain 658(Figure 5-37-D). *C. sakazakii* ST4 was the highest inducer and upregulated this gene by about 16835 times greater than the expression in non treated cells (Figure 5-37-A) followed by *C. sakazakii* strain 1557 that showed about a 4850-fold increase. The third highest induction was by *E. coli* K1 with a 4240-fold increase compared to untreated cells(Figure 5-37-B). *C. malonaticus* strain 1569 inceased the expression by about 200-fold compared with untreated cells (Figure 5-37-C). Treating with *C. malonaticus* resulted in upregulation of this gene by about 37-fold compared to the untreated cells. Other strains increased the gene expression with a range of 6-9-fold.

NF-kB is known as the key regulator of inflammatory response and cytokine production. Most of the bacterial isolates upregulated thegene encoding subunit 1 of the protein in

H4 cells. *E. coli* K1 showed the maximum stimulation, and the expression of this subunit in response to this strain was increased by about 8-fold compared with non-treated cells. Only two *Cronobacter* isolates showed a minor decrease in the gene expression which were *C. turicensis* 1211 and *C. sakazakii* strain 709. *C. malonaticus* strains 1546, 1569 and 681 upregulated the expression by about 4, 3 and 2.5-fold respectively (Figure 5-38). *C. sakazakii* ST8 strain 1 increased the expression by about 4-fold compared to untreated cells, while the rest of the strains showed less than a threefold increase.

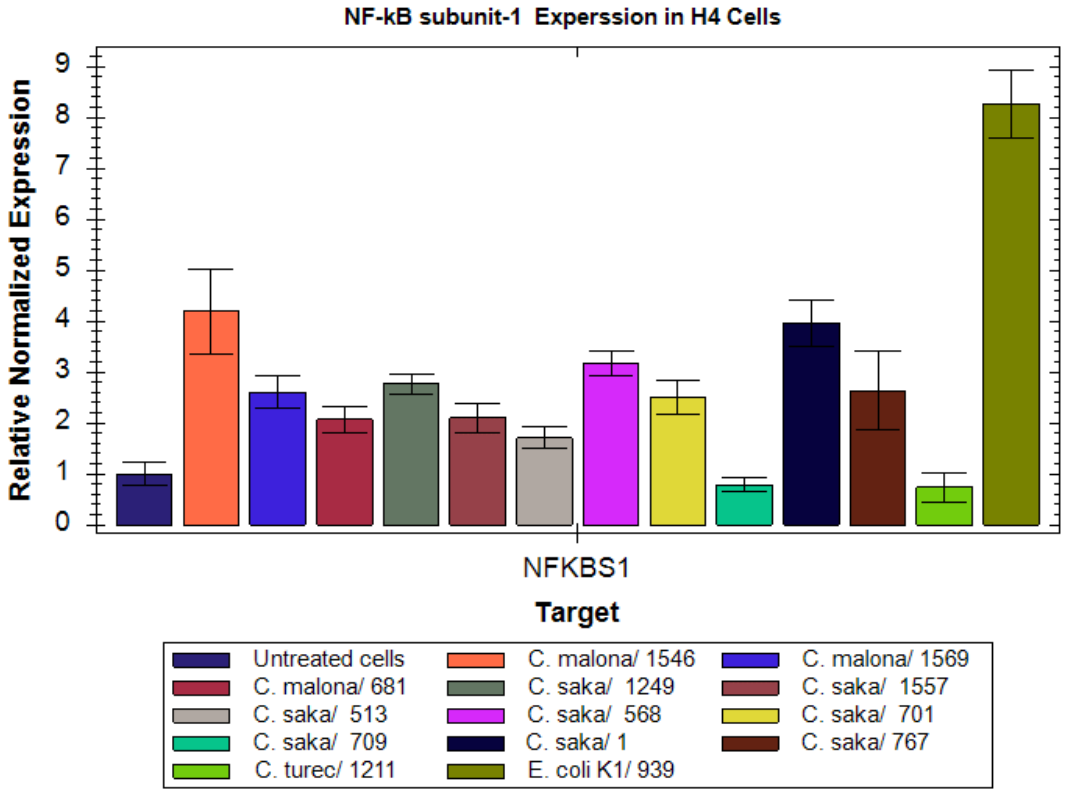


Figure 5-38 NF-kB subunit-1 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The effect of bacterial isolates on NF-kB subunit-1 expression in Caco-2 cells was different to that shown by H4 cells. All of the *Cronobacter* isolates showed slight suppression of this subunit expression in this cell line compared with untreated cells with exception of *C. sakazakii* strain 1 which did not show any effect on the expression of subunit 1. NF-kB subunit-1 in Caco-2 cells was only upregulated in response to *E. coli* K1.

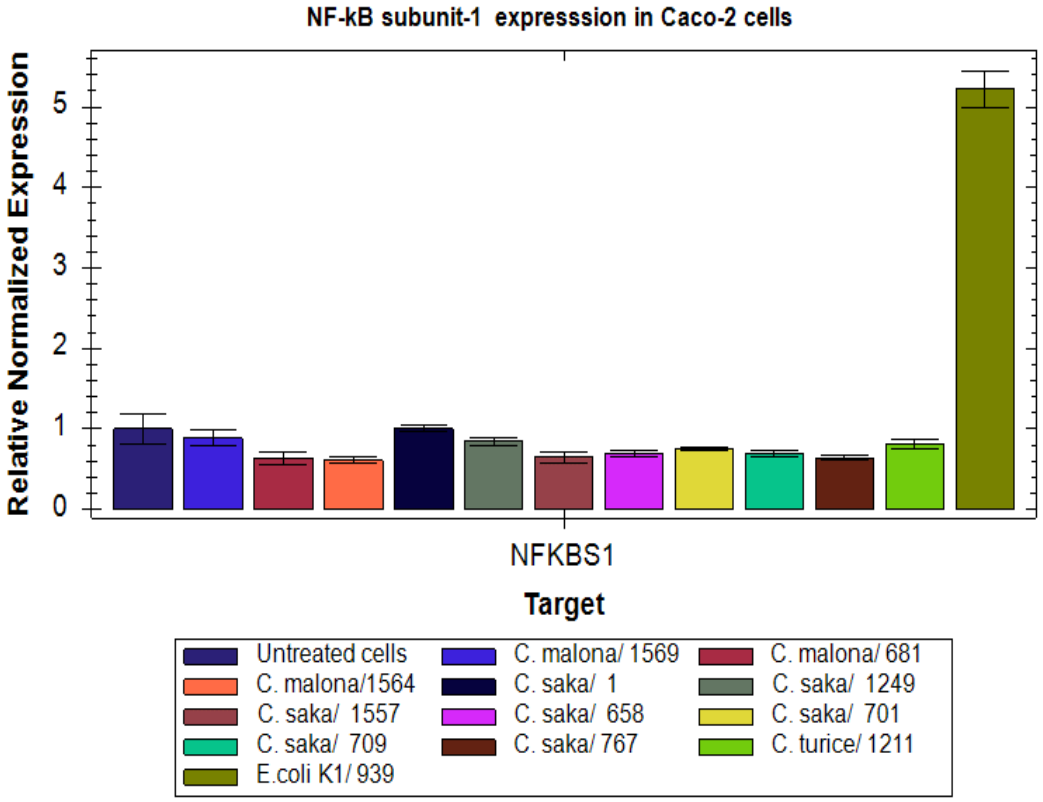


Figure 5-39 NF-kB subunit-1 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

The expression of NF-kB subunit-2 in H4 cells in response to bacterial activation was different to subunit-1. More than 76% of bacterial strains downregulated the expression of this subunit, while only two strains upregulated the expression, *C. sakazakii* strain 767 (1.6-fold) and C. turicensis (1.2 fold) whereas no effect was seen for *C. sakazakii* strain 709. Treating with E. coli K1 resulted in markedly suppression by about 90% of the expression in untreated cells (Figure 5-40). C malonaticus strains downregulated this subunit by 30% to 70% of control. *C. sakazakii* strain 1249 showed about 80% suppression while other *C. sakazakii* strains down regulated the expression by 50% or less.

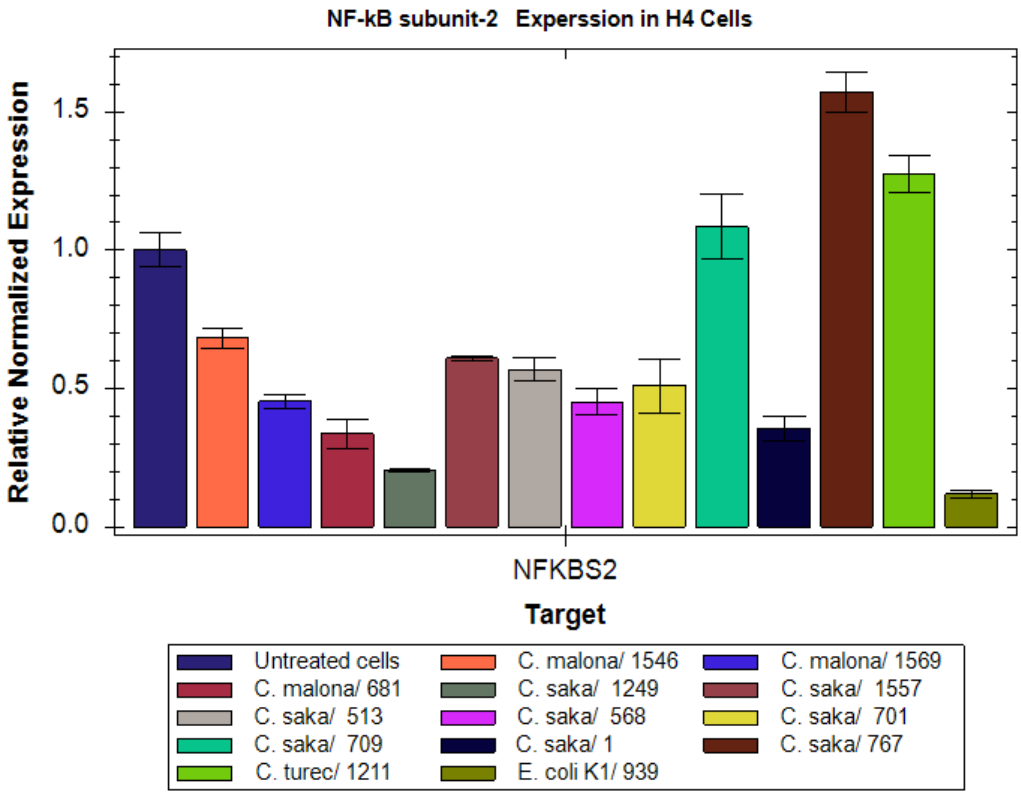


Figure 5-40 NF-kB subunit-2 expression by H4 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph.

Caco-2 cells was more responsive to bacterial activation in regard to NF-kB subunit 2. All strains upregulated the expression of this subunit, and the highest increase was about 60-fold shown by *E. coli* K1, followed by, *C. sakazakii* strain 709 that upregulated the expression of NF-kB subunit 2 about 14-fold (Figure 5-41-A). Incubation of this cell line with *C. sakazakii* strain 1557 resulted in increased expression of about 6-fold higher than the untreated cells. The upregulation resulting for incubation with *C. malonaticus* strains ranged from about 1.5 to 2.5-fold. About 2.5-fold increase was obtained by *C. sakazakii* strains 1 and 1249, while *C. sakazakii* ST4 strains 701, 709 and 767 showed only 1.7-fold increase. Threefold increase in the expression was obtained when cells were co-cultured with *C. turicensis* strain 1211 (Figure 5-41-B).

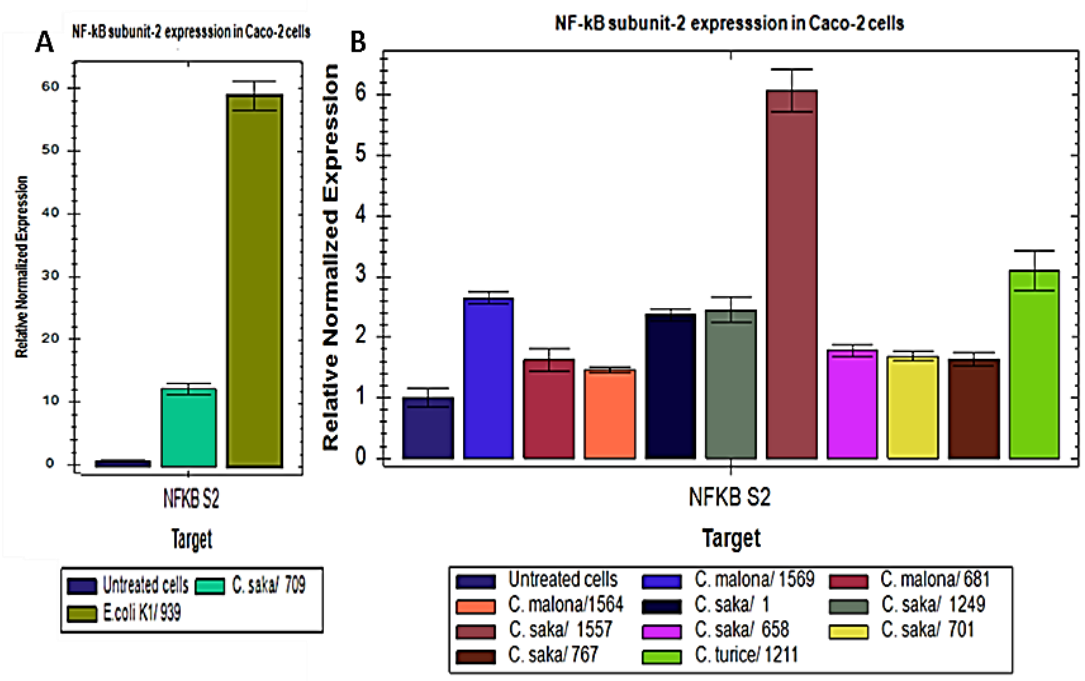


Figure 5-41 NF-kB subunit-2 expression by Caco-2 cells co-cultured with selected bacterial isolates. Total RNA was extracted 3h after initiation of co-culture, and expression levels of TLR2 were analysed by RT and qPCR. Each sample was analysed in quadruplicates and data are expressed as mean± standard errors. Results were normalized with β–actin and the expression levels of untreated cells were set as 1. Strains legends arranged from left to right according to their position in the graph. **A)** *C. sakazakii* strains 709 and *E. coli* that highly upregulated the TLR10 expression in Caco-2 cells (12and 60-fold respectively). **B)** Strains that induced the expression of NF-kB subunit 2 in caco-2 cells fro 6-fold or less.

Data on effects of co-incubation of selected bacterial isolates with Caco-2 and H4 cells on the TLRs expression is summarised in tables 5.1 and 5.2 and shown as a heatmap.

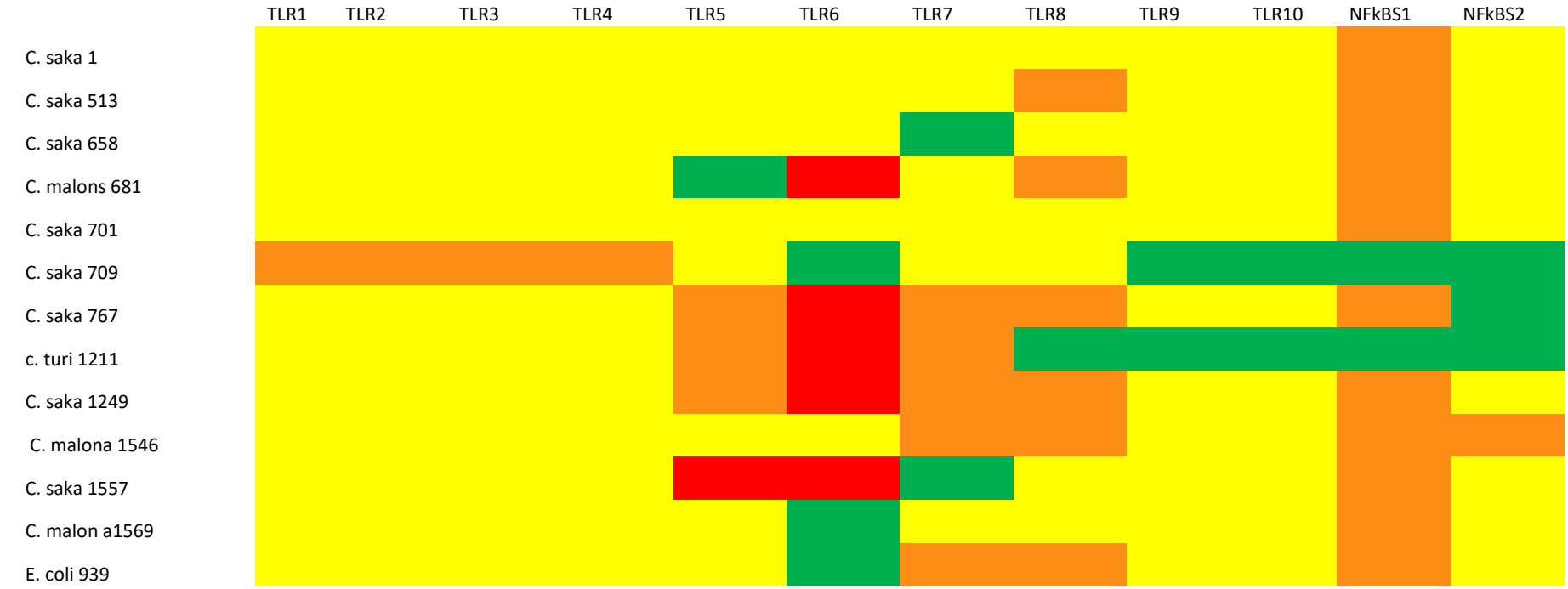


Figure 5-42 Summary of the effect of bacterial isolates on the expression of the investigated genes in H4 cell line shown as a heat map.

Down regulation <0.75 fold of untreated cells

No effect 0.75-1.5 fold of untreated cells

Slight upregulation 1.5-10 fold of untreated cells

High upregulation 10-50 fold of untreated cells

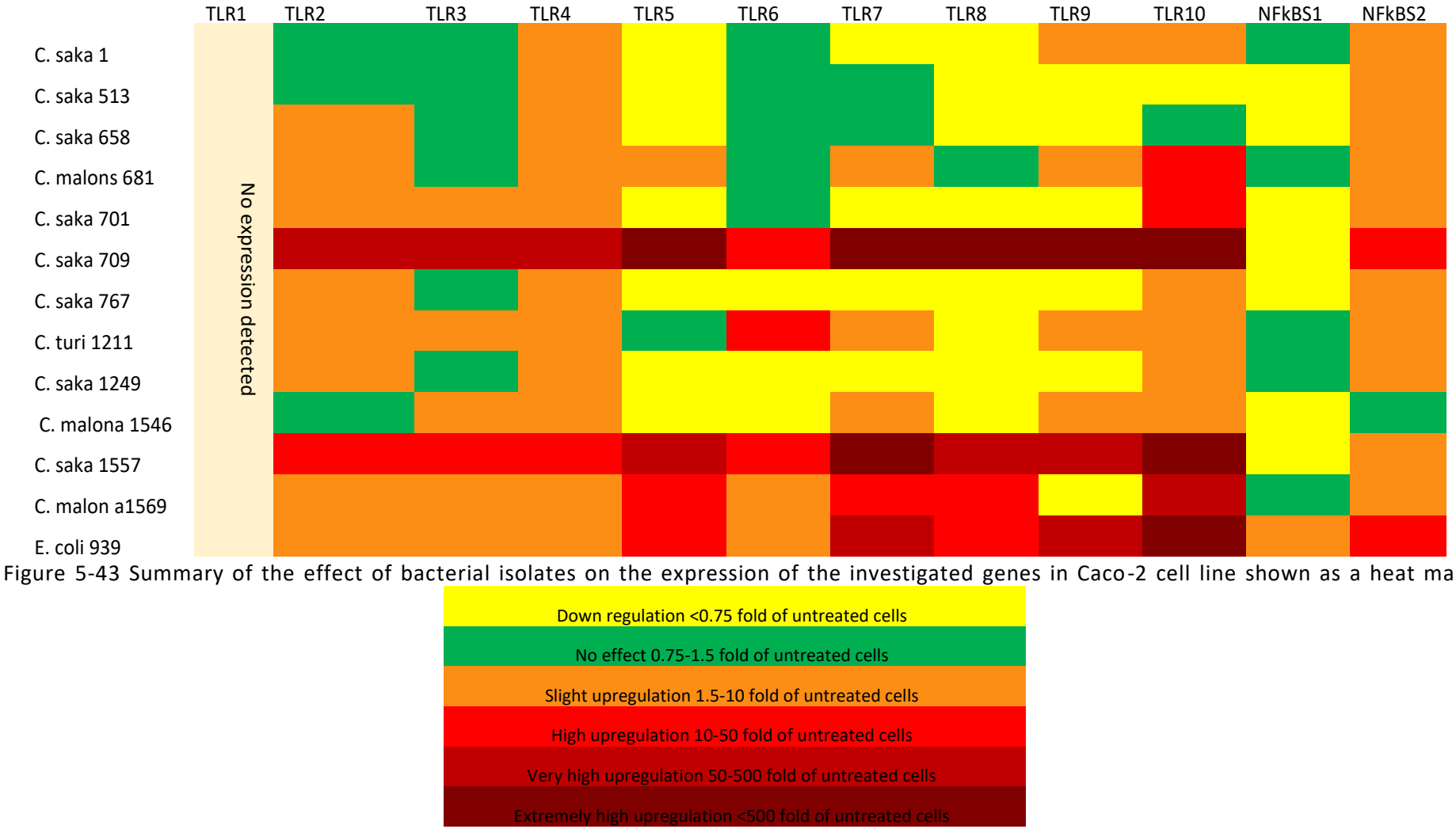


Figure 5-43 Summary of the effect of bacterial isolates on the expression of the investigated genes in Caco-2 cell line shown as a heat map

5.5 Discussion

Activation of the host immune system is the key factor in fighting disease. Host cells are provided with variable signaling proteins that function to detect and identify the foreign antigens and microbial infection. These proteins called pattern recognition receptors (PRRs) can be found on the cell surfaces and intracellularly on the endosomal membranes. These PRRs are varied in their function and structure as well as the reaction process and as an example of these PRRs the Toll-like receptors has been well characterized and investigated. When antigens are detected by these PRRs, complex signal pathways are activated and multiple inflammatory mediators are produced including pro/anti-inflammatory cytokines, chemokines and growth factors leading to clearance of invading bacteria. However, over amplification of the initial host response could result in inflammatory diseases that lead to host tissue damage and severe complications (Van Avondt *et al,* 2015).

5.5.1 Enzyme-linked immunosorbent assay (ELISA) detection method to evaluate IL-8.

Co-culturing of different bacterial strains from genus *Cronobacter* (n=25) and one isolate of *E. coli* K1 with neonatal intestinal epithelial H4 cells as well as Caco-2 cells demonstrated that both cell lines were able to produce variable amounts of IL-8, other cytokines and inflammatory mediator s investigated in this chapter. H4 cell line is a non-malignant cell line derived from small intestinal epithelium of 20 to 22 weeks gestation foetuses (Sanderson *et al,* 1996), and was used as a new model for immature small intestinal epithelial cells to comparison with Caco-2 cells.

5.5.1.1 IL-8 production non-polarized monolayers

Using ELISA technique, all strains showed variable levels of IL-8 production by H4 cells ranging from 220 pg/ml for *C. sakazakii* strain 513 to about 5880 pg/ml for by *C. sakazakii* 767 which was isolated from neonatal fatal meningitis with significant differences between the investigate stains ($P < 0.0001$) using GraphPad Prism and one-way ANOVA/Tukey post analysis. However, prolonged inflammatory response is described to facilitate bacterial infiltration to host tissues due to increased cells

permeability and loss of integrity (Hunter *et al.,* 2008; Al-Sadi *et al.,* 2009). Although *C. sakazakii* strain 513 showed the highest growth in tissue culture medium, indicated by the increased bacterial number in the tested medium compared to other strains, it showed the lowest induction of IL-8 by H4 cells suggesting that cell properties are more important than cell numbers. This strain was non-motile, low biofilm producer and shown low invasion in H4 cells line. Flagella and pili are considered the motility organelles, aids in bacterial attachment and invasion and are also known to trigger cells response and induce IL-8 (Cruz-Córdova *et al.,* 2012).

Clinical strains from cases with serious pathologies like NEC and meningitis were among the highest inducers of IL-8 production compared with other strains ($P< 0.001$). These strains were able to stimulate H4 cells to produce up to 88 foldsmore IL-8 compared to cells incubated with FBS free medium only. *C. sakazakii* strains 701 and 767 both from ST4 and isolated from fatal neonatal infections, which are NEC and meningitis respectively, and induced the highest stimulation among all of tested strains as shown in (Figure 5. 1). This suggests that IL-8 production in this model may be a good measure of pathology.

Interestingly, blood isolates such as *C. sakazakii* strain 709, *C. turicensis* strain 564 and *C. malonaticus* strain 1569 displayed moderate stimulation of IL-8 production by H4. This observation was also shown by *E. coli* K1 strain 939, the serotype that is highly linked to neonatal infections especially meningitis. IL-8 plays an essential role in activation and triggering of neutrophils and macrophages during infections to improve bacterial clearance, and it also enhances the production of bactericidal compound by macrophages such as nitric oxide (Sahoo *et al,* 2011).

These results support the previous suggestion that some pathogenic bacterial strains have developed different mechanisms to escape/or exploit or downregulate host responses and/or using cell components such as actin microfilaments to improve their survival inside the host cells and facilitate translocation to deeper tissues (Mittal *et al.,* 2009b; Bateman and Seed, 2010; Cooper *et al.,*2011; Emami *et al* 2011*;* Mahdavi *et al.,*2013).

However, no clear correlation was observed between IL-8 production and adhesion, invasion or cytotoxicity. H4 response to the tested strains was variable even among

same ST (Figure 5-1), indicating possible variation of bacterial strategies in activation of host immune response and disease causation. A link was observed between motility and IL-8 production as shown by ST8 strains where the motile strain 1 showed high levels of IL-8 induction compared with non-motile strain 513. Interestingly, the H4 cell line showed a considerable tolerance towards bacterial LPS as indicated by low IL-8 production, and moderate response to human IL-1β (Figure 5-1). This however, could be due to the immaturity of neonatal immune system (Simon *et al,* 2015), that can be easily avoided by several pathogens.

Generally, H4 cells revealed more response to *C. sakazakii* strains than other species. This result is in agreement with previous finding suggesting that this particular species is more associated with neonatal infection (Caubilla-Barron *et al*., 2007, Giri *et al*., 2011). *C. malonaticus*, is more likely linked to adult infections (Holý and Forsythe, 2014), particularly in elderly and immunocompromised adults, and showed moderate IL-8 induction by H4 cells. The fatal meningitic isolate *C. malonaticus* 1569 produced only about 1100 pg/ml IL-8 compared with *C. sakazakii* ST4 strain 767 that is thought to be responsible for neonatal death due to meningitis and induced about 5500 pg/ml compared to nontreated cells. The high IL-8 induction by H4 cells supports the suggestion that over stimulation of this cytokine is more likely responsible for NEC.

The adult model Caco-2 cells behaved slightly differently, and maximum IL-8 produced by this cell line in response to co-culturing with the bacterial isolates was about 2200 pg/ml as shown by *C. sakazakii* ST3 strain 978 and the meningitic *C. malonaticus*, strain 1569, which is about twofold less than that shown for H4 cells untreated with this strain. This finding indicats that adult Caco-2 cells are more tolerant towards bacterial infection possibly to avoid any over stimulation that could result in chronic or immune disorders, which is in contrast to neonatal H4 cells where the development of immune system is limited. Moreover, the IL-8 produced in response to *C. sakazakii* ST4 and ST12 which are predominantly associated with neonatal meningitis and NEC in neonates respectively (Joseph and Forsythe 2011; Kucerova *et al.,*2011; Forsythe *et al.,*2014) was about half of that displayed by H4 cells. Although, Caco-2 cells were more responsive to *E. coli* K1 isolate than H4, the maximum recovered IL-8 by this technique was less than 750 pg/ml, which is comparatively lower than for most of the *Cronobacter* isolates. However, this isolate showed low invasion and high attachment to both cell lines in comparison with

strains inducing high IL-8 production, which might be because of the ability of this strain to manipulate/or regulate cell responses to improve their survival and replication.

Results obtained from this assay help to contribute better knowledge to the reasons behind the susceptibility of newborns and preterm neonates to severe illness and disease complications compared with adults especially that linked to *Cronobacter* infections. Herein, *C. sakazakii* and mainly ST4 group revealed more stimulation to produce IL-8 by H4 cells. However, cells maturity could play an important role in this process, as the lack of host cells to recognise and control foreign microorganisms with inappropriate host response could result in pathogenic bacteria overcoming host cells, and in some cases overstimulation of immune system. This could cause auto-immune diseases that might themselves lead to damage to host tissue and chronic complications such as NEC. These damaged sites of host epithelial cells could also be used as a site of diffusion to underlying tissues.

5.5.1.2 IL-8 production by non-polarized monolayers in response to bacterial OMP

Bacterial OMPs are considered important structures of the bacterial outer membrane, and coded by about 3% of the Gram-negative genome (Wimley, 2003) and 4% in *Helicobacter pylori* (Yamaoka, 2006). Membrane proteins of *Helicobacter pylori* such as OipA have been found to induce IL-8 production in gastric epithelial cells (Yamaoka, 2006). Previous studies have reported the role of *Cronobacter* flagella (Cruz-Córdova *et al,* 2012), and *C. sakazakii* outer membrane vesicles (Alzahrani *et al,* 2015) in the inflammatory response, but for the first time *Cronobacter* OMPs have been investigated for their role in inflammatory response in this research. However, in the present study the used OMPs were partially purifird and it is not unexpectesd to containe bacterial LPS that might also cause increased IL-8 induction. Incubation of human epithelial cells with purified OMPs showed that both cell lines responded and released pro-inflammatory IL-8. OMPs of *C. sakazakii* and *C. malonaticus* were the highest stimulants to H4 cell lines (Figures 5-7 and 5-8). However, no correlation was observed with the induced IL-8 and pathology history of investigated strains.

As mentioned previousely, the limitation of this assay is lack of specific OMPs identification, so high induction cannot currently be linked to specific OMPS, as well as the chance of LPS contamination cannot be excluded.

These results show a possible evidence that bacterial OMPs might play important roles in stimulation of host immune response and Induce inflammatory cytokines production. This suggestions is need to be evidenced by purification of bacterial OMPs and repeating this experiment. Moreover, neonatal epithelial cells expressed high sensitivity to bacterial OMPs compared with adult cells. This finding clearly support the sensitivity of neonatal immune system to foreign antigens and its limitation to control the inflammation. This finding is strongly suggest that using of H4 cell line and other neonatal cells to investigate host pathogen interaction and inflammatory disease in neonates is more accurately in predict bacterial virulence than adult derived cells such as Caco-2 cells.

5.5.1.3 IL-8 production by polarized monolayers

Success of pathogenic bacteria to invade and/or translocate to underlying tissues without stimulation of host response might be one important factor contributing to development of serious illnesses such as bacteraemia and meningitis, without signs such as NEC. On the other hand, the over stimulation of host immune system and exaggerated inflammatory response might increase the cells permeability, and subsequently facilitate bacterial transition and spread to other parts of host body (Eutamene. 2005). The differences in host cell response according to site of infection was investigated using polarized intestinal epithelial H4 and Caco-2 cells.

Results indicated that both cell lines exhibit a bidirectional inflammatory response based on the site of infection or activation. (See section 5.3.2 for the results). IL-8 secreted in the basolateral site after one hour incubation was much lower than the apical site ($P < 0.001$). Secretion of IL-8 from basolateral membrane plays a role in the triggering of neutrophils, macrophages and T-cells from the blood stream to sites of infection or tissue injury (Arnott *et al,* 2001; Persson *et al,* 2015), which will help in bacterial clearance from infected tissues. It is possible that the investigated strains may use mechanisms to avoid triggering immune cells to maintain better survival and

multiplication, which will increase the chance of bacterial infiltration between cells into underlying cells. However, inflammation also plays an important role in increasing of intestinal permeability (Ulluwishewa *et al.,* 2011), and facilitating bacterial translocation into deeper tissues.

This finding is in agreement with Sonnier *et al,* (2010), who reported that Caco-2 cells level of IL-8 production depended upon the site of stimulation, and that cells stimulated apically produced more IL-8 in apical chamber, while stimulation from the basolateral membranes showed similar quantities of cytokine production on both sides. Rossi *et al,* (2013) investigated the IL-8 secretion through activation of human TLRs in human intestinal cells and reported that IL-8 is produced in higher amounts by apical membranes of intestinal cells in response to triacylated lipopeptide (Pam3CSK4) and diacylated lipopeptide (Pam2CSK4) that induce IL-8 production through activation of TLR2/TLR1 and TLR2/TLR6 respectively, as well as bacterial flagellin which is known to act via TLR5. Their study indicated that TLR2 and TLR5 are more associated with IL-8 production (Rossi *et al,* 2013). (Data to be further compared with TLRs expression data).

Compared to 1h, there was an increase in the amount of produced IL-8 in upper($P<0.01$) and lower chambers observed ($P<0.0001$) after 3h incubation, and some strains induced IL-8 production in thr basolateral side more than the apical side (figure 5-4) including the *E. col* K1 isolate. However, this occurred in parallel with the increase of detection of translocated bacterial cells, which could lead to basolateral stimulation of the H4 cells. Interestingly, the produced IL-8 in the upper chamber in response to *Cronobacter* isolates that are linked to meningitis or isolated from blood was still higher than the basolateral chamber, although the translocation of these strains (CFU/figure 4-9) was much higher than those that induced more IL-8 in the lower side after 3h incubation.

For example, *C. sakazakii* ST4 strain 701 which is a fatal NECIII isolate induced 430 pg/ml and 855 pg/ml in upper and lower chambers respectively and revealed 0.05% translocation of the inoculum after 3h, which is the same time of collection of the IL-8 samples. The possible meningitic isolate from same group of ST, strain 767 revealed IL-8 induction of 868 pg/ml and 600 pg/ml in upper and lower chambers respectively and revealed 1.85% translocation of the inoculum, which is about 36-fold higher translocation than 701. Increase in inflammatory cytokines at the basolateral surface

can induce immune cell migration which will help in bacterial clearance and at the same time it may increase the self-damage. This is matching the pathology of 701 that isassociated with NECIII and caused neonatal death. However, paired t test showed no significant differences between the concentration of IL-8 inapical and basolateral chambers at 3h (P= 0.5821).

Caco-2 cells exhibited similar results and IL-8 was released apically more than basolaterally at both time points with significant differences at 1h ($P < 0.01$) and at 3h ($P<0.0001$). The bronchial secretion strain *C. sakazakii* ST23 strain 1557 was the highest inducer of IL-8 production at 1h followed by meningitic *C. turicensis* 564 (figure 5-5). Response of Caco-2 cells to bacterial infection was different to H4 cells response. Despite the increased IL-8 production in both chambers after another 2hrs, Caco-2 cells showed more response to bacterial strains on the apical sides ($P< 0.0001$) at 1h and 3 h, as indicated by the differences in IL-8 detected in the two chambers. However, the high IL-8 production in the upper side compared with basolateral side is broadly in agreement with Rossi *et al,* (2013) who hypothesized that IL-8 receptors are most likely expressed on the apical membranes of Caco-2 cells.

Nevertheless, the increase in the IL-8 production in the basolateral side shown in the present study could also result from basolateral stimulation by the translocated bacteria. However, in this assay there is no a clear link between translocation and IL-8 produced by either H4 or Caco-2 cells, and for example, the meningitic *C. turicensis* strain 564 was the highest translocated strain yet one of the lowest IL-8 inducers in basolateral side. Moreover, regarding Caco-2 cells, *E. coli* K12 which did not show any translocation was the second highest IL-8 inducer in the basolateral side (see section for 4.3.3 translocation and section 5.3.2 for IL-8 in polarized cells).

These results suggests that the adult responses including adult derived cell lines can control their response towards the foreign stimulants and mount a suitable response including production of pro-inflammatory cytokines, whereas neonates and premature infant have less ability to control infection and trigger microbial clearance by producing an appropariately immune response. This could be one of the possible reasons behind the high susceptibility of neonates to bacterial infections and serious complication such as NEC compared with adults.

However, in term of clinical history and diseases caused by these strains, when data of IL-8 produced after bacterial co-culture were collected and compared it indicated that although the amount of IL-8 produced by H4 cells was significantly higher than that displayed by Caco2, only three clinically significant strains were among the top ten IL-8 inducers for H4 cells, compared with six clinically important strains among the top 10 inducers by Caco-2. No link was found between the clinical history and the produced IL-8 in response to bacterial OMPs.

This assay presented clear evidence to the differences between adult and neonatal cell lines, and H4 cells might be a better model to investigate neonatal immune and inflammatory diseases during bacterial infection. In addition, H4 cells showed a clear link between motility in *C. sakazakii* and IL-8 induction as observed for ST8 motile strain 1 and non-motile strain 513.

We further observed a change in IL-8 production pattern between polarized and non-polarized cells. In non-polarized H4 cells, the maximum produced IL-8 was obtained by *C. sakazakii* ST4 strain 767 which was about 5000 pg/ml compared with only 885 pg/ml in polarized cells. In contrast, *C. sakazakii* 1557 ST23 was among the lowest IL-8 inducers in non-polarized H4 cells and showed the highest IL-8 induction by H4 cells after 3h incubation. And among ST4 isolates, strain 709 was the lowest inducer in non-polarized monolayers and the highest inducer in polarized monolayers, whereas the highest IL-8 inducers in non-polarized monolayers Caco-2 cells were also the highest in polarized cells such as *C. sakazakii* 1557 and *C. malonaticus* 1569. Results obtained from polarized H4 cells showed a better match to the translocation assay data as the amount of produced IL-8 increased with the increase in translocated bacterial cells, which was not shown by Caco-2 cells. This observation need further investigation using a wider range of strains from different bacterial genera. Nonetheless, to gain better understanding of neonatal immune system reaction we suggest to investigate the role of basolateral activation by bacteria on cytokine production using H4 cell lines and any available neonatal cell lines (and different adult cell lines), to compare the results with apical activation. This could include investigation of the re arrangement of host membrane proteins including the changes in the expression of host receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs).

5.5.2 Magnetic 12-Plex Luminex platform detection method

To build up better knowledge about the human response to *Cronobacter* and infection by other pathogenic bacteria such as *E. coli* K1, and how these pathogens activate or supress host immune system, we investigated the production of different inflammatory mediators including cytokines, chemokines and growth factors. These mediators are: EGF, IFN-γ, IL-1β, IL-10, IL-12 (p40/p70), IL-2, IL-4, IL-6, IL-8, IP-10, MCP-1 and TNF-α, and were chosen due to their important role in host response to microbial infections or tissue injuries.

However, results obtained from this assay were unexpected, and as an example the amount of detected IL-8 by this method was much higher than that shown by traditional ELISA although the sample used in magnetic assay was amongst the replicates used in traditional ELISA. In addition, the detection of IL-8 induced by bacterial LPS and human IL-1β was repeatedly low by traditional ELISA, while in some case it was the highest by magnetic method. This result confirmed three times. Despite the high variation between the obtained results, and due to the high expense of the magnetic bead assay we were not able to repeat the experiment for further times. However, as many publications have presented their results without including the standard deviation, we used the results obtained from the third experiment because: (1) the samples used in this assay was amongst the replicates used in traditional ELISA and we expected to get comparable results, and (2) in the first two experiment samples were diluted twice but in the protocol required only one dilution. Accordingly, statistical analysis were performed to find out if there any significant differences between the two cell lines, and not between strains.

However, host response to microbial attack is a complex process including detection of the PAMPs/DAMPs, activation of immune system, production of appropriate inflammatory mediators, and regulation of the host reaction to avoid overstimulation that could result in immune disorders (Belkaid and Hand, 2014). IL-1 beta is one of the key pro-inflammatory cytokines, that is normally produced after detection of PAMPs and/or DAMPs in inactive form called pro-interleukin-1β (pro-IL-1β), and is subsequently activated by caspase-1 enzyme, which in turn is activated by inflammasome proteins (Netea *et al,* 2010). IL-1β is vital in protection from pathogens via activation and

triggering of immune cells and enhancing production of pro-inflammatory cytokines (Sahoo *et al,* 2011).

Results indicated that H4 neonatal cells are more responsive to bacterial infection than the adult Caco-2 model (P< 0.001) (figure 5-7), and only one *C. sakazakii* strain 1249 was able to induce IL-1β by Caco-2 cells which is a meningitic isolate. Interestingly, all of bacterial strains enhanced IL-1β production by H4 cells with exception of *C. sakazakii* ST8 isolates strains 1 and 513. Both of these strains showed moderate attachment and low invasion to H4 cells compared with other strains. However, this might be because this cytokine is not expressed in normal culture conditions by these cell lines or that the IL-1 receptors (IL-1r) of both cell lines did not detect this particular sequence type.

Hunter *et al,* (2008) reported that *Cronobacter* spp. were able to induce production of several pro-inflammatory cytokines including IL-1β by rat intestinal epithelial cells (IEC-6). Inducing of IL-1β production by bacterial infection could be a possible reason for the increased susceptibility of neonates to different immune illness compared with adults. This could be due to the over stimulation of immune system via enhancing production of IL-1β in high amounts, which subsequently activates the production of different inflammatory cytokines. In addition, IL-1β is responsible for induction of Inducible nitric oxide synthase (iNOS), which might lead in increased nitric oxide (NO) production in inflamed tissues, which could then affect the cells integrity and increase their permeability via disruption of tight junctions formation and contribute to apoptosis (Chokshi *et al.,* 2008).

The response of both cell lines to the controls used in this assay was also different. H4 cells revealed IL-1β production 2-fold higher than Caco-2 in response to commercial bacterial LPS from *E. coli*, which on one hand will increase the activation of host response toward the invaded microorganisms, and on the other hand means there may be greater ability of adult cells to control their response and tolerance to microbial detection. IL-1β was used in this assay to investigate its role in activation of immune response in H4 cells and Caco-2 cells and the differences between them. Caco-2 revealed undetectable amounts of IL-1β in response to activation with 1 ng/ml IL-1β, while H4 showed about 75 pg/ml.

In general, H4 cells produced inflammatory cytokines to a much higher degree than that shown by Caco-2 cells (P< 0.01), which further supports the hypothesis of the limitation of immature cells to control their inflammatory responses to invaded pathogens.

H4 cells produced up to 20-times more IL-6 than that produced by Caco-2 cells which is considered as a significant difference (P< 0.01) (Figure 5-8). The highest IL-6 produced by H4 cells was in response to *C. sakazakii* ST23 strain 1557 (2579 pg/ml) which is a bronchial secretion isolate and showed the highest invasion to H4 cells line and among the highest for Caco-2, and also *E. coli* K1 ST95 strain 939 (2580 pg/ml) which is a neonatal enteral feeding tube isolate and showed low invasion to both cell lines, but very high attachment to H4 cells. These two strains showed only 400 and 100 pg/ml IL-6 production by Caco-2 cells respectively.

Although, there is no enough data to compare with H4 cell line, similar results have been obtained using other neonatal cells. For example, Karlsson *et al,* (2002) investigated the effect of different bacterial isolates including *Escherichia coli, Neisseria sicca* and *Pseudomonas aeruginosa*, on the immune response of mononuclear cells from neonatal cord and adult blood cells, and found that the production of IL-6 was significantly higher in neonatal samples. There are some reports indicating on association of increased IL-6 production with high mortality rate in patients with sepsis due to septic shock in adults (Hack *et al,* 1989; Calandra *et al,* 1991), and children (Sullivan *et al,* 1992). These are very important findings, as the mortality rate due to septic shock among neonates is higher than adults (Kermorvant-Duchemin *et al,* 2008). Understanding the reasons behind these differences in cell line responses will contribute better understanding to the disease development, which will help to offer suitable treatment. However, *C. sakazakii* ST4 strain 709 that was thought to be responsible for neonatal septicaemia was not the highest IL-6 inducer by either cell line.

Similarly, there was a major difference in released IL-8 (P< 0.0001), and H4 cells showed up to 10-fold higher production than Caco-2 cells (Figure 5-11) which is generally in agreement with ELISA detection method despite the change of assay method. In agreement with these results, Nanthakumar *et al,* (2000) investigated IL-8 production by H4 and Caco-2 cells in response to stimulation with bacterial LPS and human IL-1β,

and their results indicated that IL-8 produced by H4 cells was significantly higher than that released by Caco-2 cells.

In the magnetic beads assay, the stimulation of cell lines with bacterial LPS showed high inflammatory response and the highest amount of IL-8 produced, which is unexpected as indicated previously. *C. sakazakii* ST8 strains 1 and 513 showed the lowest IL-8 production by Caco-2 cells, which was also noted with IL-6, and only the ST8 did not induce any Monocyte Chemoattractant Protein-1 (MCP-1) production by this cell line. However, although this sequence type is more associated with clinical sites than other sources, it does not associate with severe infections such as meningitis and NEC (Joseph *et al,* 2012). However, *C. sakazakii* ST8 strains induced different levels of cytokine production, especially with H4 cells. *C. sakazakii* 513 is a non-motile strain and induced less cytokines production such as for IL-4, IL-6 and IL-8, compared with motile strain 1 from same group of ST, which is could be because of a possible lack of flagella in this strain. This would need to be proven by visualisation of presence or absence of flagella by electron microscopy. Stimulation with IL-1β was very low and IL-8 induction in Caco-2 cells 252 pg/ml compared with more than 6000 pg/ml produced by H4 cells.

Monocyte chemoattractant protein 1 (MCP-1) is an essential chemokine that helps in bacterial clearance via activation of immune cells such as monocytes and macrophages (Deshmane *et al,* 2009). Both cell lines produced detectable amounts of this inflammatory mediator with the exception of Caco-2 cells in response to ST8 strains. In general, there was no clear pattern of the MCP-1 production by H4 cells and production ranged between 165 to 286 pg/ml. As mentioned before, ST8 showed no stimulation of Caco-2 cells and the non-motile strain 513 was the second lowest inducer for H4 cells, despite the high level of adhesion to H4 cells, suggesting that the lack of flagella as a PAMP is possible reason for lack of cytokine induction. Strains associated with serious infections such as *C. sakazakii* strains 709, 767, 1249, and *C. malonaticus* 1569 showed high induction of MCP-1 production. MCP-1 plays important role in regulation of inflammation, and treatment with this pro-inflammatory chemokine is an attractive strategy in clearing bacteria, and promoting wound healing (Wood *et al,* 2014). Haller et al, (2000) showed that activation of Caco-2 cells with non-pathogenic *Lactobacillus sakei* and *E. coli* induced the production of different cytokines including of IL-8, MCP-1,

IL-1â, while intestinal isolate of *Lactobacillus johnsonii*, showed inhibitory feedback in the inflammatory cytokines, but induced TNF-α production at mRNA levels.

To escape immune defences and translocate to deeper tissues, some pathogenic bacteria overdrive the immune system to produce inflammatory cytokines that are important in recruiting macrophages, monocytes, T-cells and dendritic cells to site of infection. This process can facilitate bacterial translocation to blood stream through increasing the inflammation that subsequently leads to increased cell permeability and tissue damage, or via persistence and multiplication inside macrophages (Dwinell *et al,* 2001; Almajed and Forsythe, 2016).

Interferon Gamma-Induced Protein 10 (IP-10) is a chemoattractant regulatory protein and is very important in activation of the immune system (Hörmannsperger *et al,* 2009; Chen *et al,* 2011). Although, both cell lines produced very low amounts of this chemokine, it can be used as a positive marker in early stages of microbial infection, and production of high amounts of this protein was linked with NEC infection in human neonates (Ng *et al,* 2007). Results shown by both cell lines indicated that the NECIII strain 701 was not the highest IP-10 inducer and while the *E. coli* K1 strain was the highest stimulant for H4 cells, the meningitic *C. malonaticus* 1569 was the highest inducer for Caco-2 cells. These suggests host responses that could differ significantly between adults and neonates, and it might be very difficult to understand neonatal immune responses via investigating adult cells.

Bacterial isolates were investigated in respect to their capability to induce anti-inflammatory cytokines including IL-4 and IL-10. No IL-10 was detected using either cell line. Suppression, or lack of activation of this cytokine resulted in increased pro-inflammatory cytokines in *E. coli* infected mice, and contributed to high mortality rate due to increased host autoimmunity (Sewnath *et al,* 2012). This result provides evidence that both *Cronobacter* and *E. coli* K1 may have suppressed the IL-10 production through unknown process, which is may partialy explain the very high amounts of pro-inflammatory cytokines such as IL-8 and IL-6 detected in this assay using ELISA method. However, previous studies suggested that the production of IL-10 is through activation of TLR-4 in human intestinal epithelial cells to generate epithelial-macrophage cross-talk which is an important mechanism in establishing of intestinal homeostasis mediated via

host-pathogen interactions (Hyun *et al,* 2015). In our study, most strains downregulated the expression of TL4 which could further suggest an association of IL-10 production with activation of TLR4 via feedback loops mechanisms, and also for the high inflammatory response of neonatal H4 cell line.

Production of IL-4 was varied, and Caco-2 cells were nonresponsive to about 38% of bacterial isolates (5/13) while H4 cells were nonresponsive for only 15% (2/13) which are the non-motile ST8 strain 513, and the bed-swab *C. malonaticus* strain 1546. This in addition to previous findings in this project is and possible further evidence to the role of flagella in stimulation of host immune system and cytokine production. However, this role might be key for virulence of specific sequence type or species, and might not be applicable for another bacterial species. For example, the non-motile *C. universalis* was able to enhance moderate IL-8 production by both cell lines. Among strains that did not enhance IL-4 in Caco-2 cells are *C. sakazakii* ST4 strain 701 that is thought to be responsible for neonatal death due to NECIII and *E. coli* K1 939 (high adhesive strain) as well as *C. sakazakii* ST1 strain 658, the non-clinical isolate, but both showed moderate attachment and invasion in both cell lines.

However, this cytokine is important for host immune regulation, and several studies indicated to the role of IL-4 in inhibiting Th1 cell development and driving differentiation of Th2 (O'Garra, 1998). Any dysregulation of this cytokine could result in immune disorders or inflammatory diseases. Selected *C. sakazakii* isolates including *C. sakazakii* ST4 strain 767 and ST1 strain 658 were found to induce IL-4 production in Human microglial cells (HMGC) and Human brain microvascular endothelial (HBMEC) cells (Almajed 2015, unpublished data).

Bacterial isolates were further investigated for their capability to enhance epidermal growth factor (EGF) production by epithelial cells. Whereas this cytokine was induced by all strains in H4 cells, it was only produced by Caco-2 cells in response to clinically significant strains and in very low amounts which is significantly lower than H4 cells($P < 0.0001$), and no enhancement was detected due to bacterial LPS or human IL-1β. This suggests that this cytokine is not induced by bacterial LPS, and it could be activated through different bacterial components such as lipoproteins, DNA or flagella.

Price and Sheldon, (2013) recorded an increase in the expression for the EGF in response to triacylated lipoprotein Pam3CSK4 (PAM) that known to activate TLR1/TLR2 heterodimers, and in addition they noted an increase in IL-6 accumulation in response to lipoproteins in presence of EGF by granulosa cells from bovine ovary. These results may explain the differences in IL-6 production between the two cell lines. H4 cells showed IL-6 production significantly higher than Caco-2 cells and in mean time, only a few strains showed low EGF induction by Caco-2 cells but two of them showed the highest IL-6 production which *C. sakazakii* 1249 and *C. malonaticus* 1569 which are both meningitic isolates (Figure 5-10 for IL-6, and 5-15 for EGF). EGF is important for normal intestinal development as well as repair following the injury of gastrointestinal mucosa (Warner and Warner, 2005), and up/or downregulation of this cytokine expression will possibly affect the normal development of intestinal cells, and might contribute bacterial entry into underlying tissues. Deficient EGF production was associated with NEC in neonates (Warner and Warner, 2005; Claud, 2009).

In addition to those cytokines discussed above, IL-2, IL-12, IFN-γ and TNF-α were also investigated. IL-2, IFN-γ and TNF-α were undetectable, while IL-12 was secreted by H4 cell line in response to only four strains, two of them clinically significant strains which are *C. sakazakii* strain 709 (septicaemia) and *C. turicensis* 1211 (meningitis). No detectable levels of these cytokines were recorded for Caco-2 cells. These results suggestes that the responses of H4 cells generally show a closer correlation with pathology associated with some strains than the response seen in Caco-2 cells.

IL-2 inhibits autoimmunity through enhancing the differentiation of immature T-cells into regulatory T cells, which suppress other mature T cells from attacking healthy cells in the body (Malek and Castro, 2010; Liao *et al,* 2011). IL-12 activates IFN-γ production by Th1 cells that activates macrophage phagocytosis, and IL-12 therapy hase been shown to increase incidence of neonatal survival and to reduce bacterial load (Orme *et al,* 1992; Flynn *et al,* 1995; Cooper and Khader 2008; Hamza *et al,* 2010; Redford *et al,* 2010). Therefore, the absence of IFN-γ could be because of the downregulation of IL-12 by pathogenic bacter. However, the suppression of regulatory IL-10 production was expected to increase release of IFN-γ and TNF-α and other pro-inflammatory cytokines (Sewnath *et al,* 2012), which indicates the potential association of IL-12 suppression with

the low expression of these two cytokines. Summarised results of detected cytokines and growth factors are shown in table 5.3.

Despite the limitation indicated previously about this assay, obtained results provide clear evidence of differences between adult and neonates cells, and suggest that H4 cells may show responses similar to those reported cases of induced inflammatory diseases and increased autoimmunity. This enhanced response may explaine the pathophysiology of some chronic illness including NEC. Additional investigations are crucially needed to clarify the mechanisms involved in the regulation of the balance between anti and pro-inflammatory responses of intestinal epithelium. In addition, it is very important to understand whether apical activation of epithelia cells is responsible for increase neonatal immune responses or if this resulted from basolateral activation by invaded or translocated pathogens. Polarised bilayers are possibly a better model to understand these mechanisms as they may more closely mimic the real situation in infection cases.

Table 5-1. Summary of detected cytokines and growth factor in H4 and Caco-2 cell lines.

bacterial strains	H4 cell line							bacterial strains	Caco-2 Cell line						
	IL-6	MCO-1	EGF	IP10	IL-4	IL-8	IL-1B		IL-6	MCO-1	EGF	IP10	IL-4	IL-8	IL-1B
1	1481.9	249.41	5.53	1.13	45.33	8204.93	0	1	158.97	45.58	0	1	2.2	212.18	0
513	678.72	176.11	12.88	1	2.2	3857.42	0	513	134.29	33.78	0	0.37	0	161.85	0
658	1363.1	286.99	12.88	0.86	53.1	10777.5	13.56	658	113.18	70.11	0	0.71	0	589.13	0
681	1279.87	275.5	19.1	1.49	60.73	8830.64	21.81	681	239.6	149.41	0	0.55	12.03	2799.43	0
701	1206.58	254.25	15.8	1.06	75.68	12256.38	28.6	701	295.59	113.22	0	0.86	0	1578.11	0
709	1143.07	254.25	15.09	0.86	49.23	8343.04	34.59	709	500.74	192.89	2.79	0.55	16.52	6113.72	13.56
767	1404.74	254.25	9.58	1	45.33	11693.14	13.56	767	329.92	164.5	2.79	0.63	12.03	4855.21	0
939	2588	286.99	5.53	1.6	104.62	13152.44	28.6	939	105.19	82.92	0	0.71	0	1002.65	0
1211	1074.24	249.41	7.69	1	75.68	9823.54	13.56	1211	359.57	170.35	0	0.93	2.2	4423.93	0
1249	1059.32	254.25	2.79	0.86	37.41	8551.04	13.56	1249	322.94	152.48	2.79	0.79	12.03	2775.64	13.56
1546	470.93	164.5	2.79	0.86	0	3381.4	13.56	1546	354.58	140	2.79	0.55	0	2600.77	0
1557	2573.6	286.99	17.15	1.25	75.68	14701.65	28.6	1557	404.08	158.55	8.66	1	20.87	3432.75	0
1569	1281	263.79	9.58	1.31	53.1	10076.78	21.81	1569	599.18	187.38	5.53	1.13	29.28	4900.84	0
BLANK	94.4	45.58	0	0.37	0	320.17	0	BLANK	4.71	33.78	0	1.06	0	207.49	0
LPS	2074.87	313.75	13.64	2.36	45.33	15195.96	28.6	LPS	2499.78	187.38	9.58	0.55	68.25	3741.58	0
IL1	535.62	214.24	5.53	1	20.87	6197.99	74.75	IL1	54.77	33.78	0	1.31	0	255.72	0

Strains within each cell line were categorized onto 3 groups from high to low production. Red indicate high production, orange represents medium production and yellow low production. Production by untreated control cells is indicated green. Actual amounts are indicated in the relevant boxes.

5.5.3 Discussion of TLRs results.

During the delivery and thereafter, neonates are exposed to dramatic changes in the surrounding environment from completely sterile to a new milieu populated with millions of microorganisms, of which some are pathogenic. In this study, the response and reaction of neonatal intestinal epithelial H4 cells to bacterial attack was investigated through determination of the changes in the expression of human Toll-like receptors (TLR1-10) and NF-kB in the presence of bacteria. Results were compared with the adult model cell line Caco-2. Neonatal H4 cell line was chosen as a new model for neonatal immunity investigation, while Caco-2 cell line was chosen because it is well characterized and its response to bacterial challenge has been previously studied. Untreated cells were used as an experimental control and the expression of these genes in this control was set as 1.

The investigation of the differences in gene response between adults and neonates is critical, and can contribute to a better knowledge of the immune regulatory response in neonates. This is crucial to improve our understanding of the disease development and potential treatment applications. TLRs are a family of membrane proteins that contribute the a role in the host immune system, and are found in the cell membrane of the host cells as well as on the membranes of some organelles such as vesicles. Information about these TLRs and their ligands are presented in table 5.1 according to Kim *et al,* (2010).

Table 5-2. Location of human TLR and their ligands according to (Kim *et al,* 2010)

Receptor	Location	Ligand	Ligand source
TLR1	Cell surface	Multiple triacyl lipopeptides	bacteria
TLR2	Cell surface	Multiple glycolipids Multiple lipoproteins Heat-hot shock protein 70 (HSP70) Zymozan	Bacteria Bacteria Host Fungi
TLR3	Endosomal	Double-strand RNA (dsRNA)	Viruses
TLR4	Cell surface	Lipopolysaccharides (LPS) Several heat-hot shock protein fibrinogen	Gram-negative bacteria Bacteria and host cells Host
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	Multiple diacyl lipopeptides	Mycoplasma
TLR7	Endosomal	Single-strand RNA (ssRNA)	RNA viruses
TLR8	Endosomal	Single-strand RNA (ssRNA)	RNA viruses
TLR9	Endosomal	Unmethylated CoG oligodeoxynucleotide DNA	Bacteria, DNA viruses
TLR10	Cell surface	?	?

TLR: Toll-like receptor, LPS: Lipopolysaccharides, **DNA:** Deoxyribonucleic acid, **RNA:** Ribonucleic acid, **COG:** Clusters of Orthologous Groups protein

The aim of this experiment was to determine whether these PRRs are involved in the detection of pathogen attack and induction of the inflammatory response. This will contribute to a better understanding of the roles of these receptors in pathogen detection and disease development, particular NEC which is mostly linked to exaggerated inflammatory response (Martin and Walker, 2006; Claud, 2009; De Plaen, 2013; Hunter and De Plaen, 2014).

Results obtained from this study indicate that bacterial isolates are able to affect the expression of these genes in both cell lines. Our results for the first time introduce evidence that almost all of human TLRs are expressed in human intestinal cells, which are the first defence barrier against the invading microorganisms, and function as sensors to detect PAMP such as LPS and activate the host immune system. The only exception was TLR1, which was not detected in both treated and untreated Caco-2 cells. Previous studies indicated a possible role of this TLR1 in IL-6 induction (DePaolo *et al,* 2012). However, Caco-2 were able to produce IL-6 in response to bacterial infection, which suggest different factors could be involved in this cytokine induction.

The expression of TLRs in H4 cells in presence of bacteria varied largely, and most of bacterial isolates downregulated TLR1-4 with exception of *C. sakazakii* ST4 strain 709 that was found to upregulate the expression of this gene. The effect of the rest of strains ranged from about 50% suppression to complete silencing of these genes. *C. sakazakii* ST8 strain 1 which is a child's throat isolate blocked the expression of TLR1, and in comparison with the inflammatory response, this strain showed high IL-8 induction in H4 cells and was among the low adhesive and invasive strains for H4 cells. Both ST8 isolates 1 and 513 showed no induction of IL-1β, one of the most important inflammatory cytokines.

Ohta *et al,* (2014) investigated the effect of human TLRs activation on IL-8 production by submandibular gland epithelial cells (SMGCs). Their findings indicated that IL-8 is multiple TLR dependant, and it was induced by a variety of TLR ligands including synthetic triacylated lipoprotein (Pam3CSK4) that is a TLR1/2 ligand, polyinosinic-polycytidylic acid (poly I:C) a TLR3 ligand, LPS (TLR4 ligand), Flagellin (TLR5 ligand), macrophage-activating lipopeptide (MALP)-2 (TLR2/6 ligands). However, the dysregulation of TLRs by bacterial infection reported in this project was weakly linked to

cytokine production. *C. malonaticus* 1546 and *C. turicensis* 1211 both showed the maximum suppression of TLR1 expression, and *C. turicensis* 1211 induced IL-4, IL-8 and EGF more than that detected in response to *C. malonaticus* 1546 as shown using the magnetic beads assay. However, both strains showed high IL-8 induction using the ELISA method.

The variation between other strains was minor, thus no link could be made between this TLR and other phenotypes. DePaolo *et al,* (2012) found that TLR1 is important for IL-6 induction by DCs, which is in contrast with the results obtained by H4 intestinal epithelia cells, where most of bacterial strains showed significant induction of inflammatory cytokines such as IL-6 and IL-8 despite the significant suppression of TLR1 and other receptors. In contrast, this receptor was not detected in Caco-2, which is in agreement with DePaolo *et al,* (2012), and might explain the low levels of IL-6 produced by this cell line which could be a result of the absence of the active TLR1 and subsequently the limitation of detection of foreign ligands. Moreover, different cell lines may express different TLRs and TLR1 may not be expressed in this cell line. Our findings show that most strains downregulate TLRs 1-4 and induce inflammatory cytokines in H4 cell line, which is in agreement with Choteau *et al,* (2017) who indicated that TLR1 plays important role in the homeostasis of intestinal epithelium and depletion of this gene results in increased intestinal inflammation.

However, TLR1 that is known to be activated by multiple triacyl lipopeptides from bacteria was undetectable in Caco-2 cells. However, this experiment was confirmed twice with same results, and further investigation is strongly advised with same strains used in this study to confirm these results.

As mentioned previously, most of the investigated strains behaved similarly with regard to the expression of TLR2, TLR3 and TLR4 in H4 cells and resulted in suppression by more than 50% of these genes. Choteau *et al,* (2017) indicated that TLR2-deficient mice displayed increased susceptibility to intestinal inflammation, which is largely in agreement with our results showing strains significantly inducing inflammatory cytokines in parallel with reduction of TLR2 expression that is thought to limit the ability of host cells to detect pathogens via this receptor, and results in increased inflammatory response (Choteau *et al,* 2017). Strain 709 that caused an increase in expression of this receptor gene, was among the low adhesive strains despite the markedly high invasion

and inflammatory cytokine induction, and completely differed from ST4 strains indicating no effect of the ST membership on the phenotype of the strain.

TLR2 was upregulated in Caco-2 cells by almost all of investigated strains with small variation, although this cell line displayed a weaker inflammatory response measured as cytokine production, which supports the study by Choteau *et al,* (2017) on the importance of TLR2 in mediumting tolerance in epithelial cells.

However, Elena *et al,* (2009) reported that TLR2 is involved in activation of NF-kB in collaboration with MyD88, which subsequently activates the production of inflammatory cytokines. However, the results presented here showe that most strains downregulated the expression of TLR2, and NF-kB subunit-2 while upregulating NF-kB subunit-1, which is more associated with increased pro-inflammatory cytokine production including IL-6 and IL-8 by both cell lines, while the downregulation of subunit 2 did not result in decreased production of these cytokines. This most likely indicates that the increased expressed mRNA of NF-kB subunit-1 is translated to an increase in the amount NF-kB subunit-1 proteine available for activation.

TLR3 more likely works as a PRR for viral double-stranded RNA (Jin and Lee, 2008), and it is up regulated in HT29 cell line only by Gram-positive commensal bacteria at the levels of both mRNA and protein (Furrie *et al,* 2005). This TLR was downregulated by bacterial challenge in H4 cells, with the only exception of *C. sakazakii* strain 709, which is in contrast with Caco-2 cells when most of the strains induced the expression of this receptor.

Ritter *et al,* (2005) investigated the role of TLRs in inflammatory response, and found that the stimulation of primary small-airway epithelial cells (SAEC) with TLR3 ligand poly(I:C) resulted in increased secretion of chemokines and cytokines including IL-6, IL-8, MCP-1, and TNF-α, while peptidoglycan (PGN) and Flagellin that are TLR2 and TLR5 activators respectively, only increased the release of IL-8. Interestingly, this cell line showed no cytokine or chemokine secretion in response to bacterial LPS (Ritter *et al,* 2005). The production of inflammatory mediators are mostly higher in H4 cells, despite the downregulation of these TLRs by most bacterial strains, which is expected to affect the presence of active protein of these TLRs, suggesting that H4 cells are more redialy

induced to develop inflammation as indicated by the high production of IL-6, IL-8 and MCP-1 compared with Caco-2**.**

TLR4 is known to detect pathogen associated molecular patterns (PAMPs) such as LPS. This receptor was downregulated in H4 by almost all of bacterial isolates by up to 99%. The suppression of this gene did not corrolate with the ability of bacterial isolates to induce high amounts of pro-inflammatory cytokines, suggesting that the incidence of high inflammatory response and immune disease in neonates may not be associated with the presence or absence of this TLR and other downregulated genes. Different factors might be involved, which require further investigation. Moreover, it has been shown that prolonged incubation of human mesenchymal stem cells (MSCs) with bacterial LPS caused downregulation of this gene (Mo et al., 2008), which is a potential explaination for the obtained results.

However, previous studies indicated the important role of MD-2 in the function of several TLRs including TLR2 and TLR4, and their response to different ligands such as LPS, which on the other hand could affect the regulation of the intestinal immune responses to commensal organisms. Primary study by Abreu *et al,* (2001) indicated that IEC do not express MD-2 which might explain why these genes are not highly expressed in these cell lines in response to bacterial challenge. Palazzo *et al,* (2008) found that TLR4 expression was downregulated in HT29 in response to LPS, flagella and peptidoglycan. In a study by Fusunyan *et al,* (2001) on the effect of bacterial LPS and IL-1β on the expression of TLR2 and TLR4 in H4 cells and in samples of small intestinal ileum from human foetuses, they found that the expression of TLR2 and TLR4 was increased by IL-1β, while LPS decreased TLR4 expression.

In contrast to H4 cells, TLR4 was induced in Caco-2 cells by all bacterial isolates. This cell line showed lower cytokine induction suggesting that the expressed mRNA of this TLR may not be translated to active protein, or that the cytokine production in adult cell lines including Caco-2 is regulated at a different level and not effected by the dysregulation of TLR expression. Moreover, this cell line is derived from colon carcinoma which means that it is genetically abnormal and may not respond normally.

The neonatal H4 cell line showed more robust response to bacterial challenge with regard to TLR5 as some strains upregulated its expression (Figure 5-26). This TLR is

expressed in a variety of cell types and is essential to initiate inflammatory responses particulary to flagellated bacteria (Akira *et al,* 2006; Feuillet *et al,* 2006). Most strains that upregulated the expression of TLR4 in H4 cells are highly motile and clinically important such as *C. turicensis* strain 1211 and *C. sakazakii* 1249, that are both associated with fatal infections, suggesting the possible role of feedback loops in this process. These strains induced moderate to high cytokine secretion.

Feng *et al,* (2012) suggested that the signalling of TLR5 in the lamina propria is essential in the maintenance of homeostasis between the host immune system and microbiota through modulation of the activity of effector Treg cell and T cell balance. Strain 1557, the most potent activator of TLR5 in H4 cells, was among the weak IL-8 inducers in non-polarized cells, but the strongest in polarized monolayers. This strain was among the lowest adhesive strains but the most invasive to H4 cells. Zeng *et al,* (2003) suggested that TLR5 is expressed in the basolateral side of epithelial cells and plays an essential role in the detection of invasive bacteria through recognition of their Flagellin, suggesting that the invasive strains may use their flagellin to regulate this receptor, which subsequently activates the immune response. However, the excessive upregulation of this TLRs by these strains may indicate to that the mechanisms behind activation and expression of this TLR can occur internally or basolaterally. *C. sakazakii* strain 1557 was among the highest translocated strain.

Regarding Caco-2 cells, TLR5 expression was upregulated by some of the investigated strains. *C. malonaticus*, which is more associated with adult infections induced this TLR more efficiently in Caco-2 cells than in H4 cells. This result indicates that different cell lines could express different responses to the same ligands, and display different levels of gene expression accordingly. *C. sakazakii* strain 709 is linked to severe neonatal septicaemia and increased expression more than 2000-fold, which may be because of the ability of this strain to upregulate the expression of TLR5 through the basolateral side of cells where this protein is present in higher amount. No clear link was observed between the inflammatory cytokine production and the dysregulation of this gene expression in Caco-2 cell line. For example, *E. coli* K1 was one of the weakest stimulants, although it was among the highest translocated strains, and displayed highly upregulated TLR5 expression. Results for this strain support our findings that the

activation of TLR5 expression might be induced via basolateral membranes and by the translocated bacterial cells.

Moreover, another possible link was observed between the upregulation of gene expression and bacterial translocation in Caco-2 cell line shown by *E. coli* K1. This strain was among the highest translocated strains, and displayed increased induction of TLR5 expression, which is supports our previous suggestion of that the activation of TLR5 expression might occur the on the basolateral membranes by translocated bacterial cells. This requires further investigation using the polarised cell line model (Gewirtz *et al,* 2001; Oppong *et al,* 2012).

TLR6 plays a key role in the detection of pathogens and activation of the immune system, with a possible role in inflammatory bowel disease development (Pierik *et al,* 2006). This TLR was dysregulated in the investigated cells by incubation with some bacterial strains. Like TLR1, TLR6 is known to form heterodimers with TLR2 (TLR6/TLR2) and act to recognise diacylated lipopeptides instead of triacylated lipopeptides which are recognized by the TLR1/2 heterodimer (Morgan *et al,* 2014). Strains that significantly upregulate the expression of this gene are historically important, most of them associated with fatal illness in neonates such as the meningitic *C. sakazakii* strains 1249 and 767 and the fatal *C. turicensis* strain 1211. Unexpectedly, this gene was not induced by the *C. sakazakii* strain 709 that was responsible for neonatal death due to NECIII, which is highly linked to inflammatory bowel disease.

Previous studies suggested that TLR2/6 heterodimer can stimulate immature dendritic cells to produce tolerogenic dendritic cells and help in the development of regulatory T-cells (Tregs) (DePaolo *et al,* 2008; DePaolo *et al,* 2012). Tregs have the ability to limit the pro-inflammatory activities of different immune cells (Pot *et al,* 2011). This signalling pathway gives TLR6 an important role in maintaining the homeostasis, and contributes in limiting immune diseases. This suggests that the dysregulation of neonatal immunity is multifactorial, and can be induced by different factors rather than the changes in the gene expression at the mRNA level.

Only *C. sakazakii* strains 1557 and 709 significantly enhanced the expression levels of TLR6 in Caco-2 cells compared with untreated control cells. This increased expression showed minor association with high secretion of pro-inflammatory IL-6 and IL-8, which

may indicate parallel translation of the expressed mRNA of TLR6 to active protein that is then able to detect of pathogens and activate the immune system. TLR6 is important for development of T-helper 17 (Th17) cells in the lung, which are particularly important for the control of extracellular pathogens such as bacteria and fungi (Moreira *et al,* 2011).

TLR7 was dysregulated by bacterial infection, despite its association with detection of viral single stranded RNA and the response to viral infection (Kru ger *et al,* 2015). *C. sakazakii* meningitic strains 1249 and 767 as well as *E. coli* K1 meningitic serotype increased the expression of this gene. In addition, to *C. sakazakii strain* 1557 demonstrated the ability to upregulatemost of the investigated genes mainly in Caco-2 cells. Although, most of these strains induced high levels of pro-inflammatory cytokines and chemokines such as IL-6, IL-8 and MCP-1, no correlation was observed between the level of TLR7 expression and the secreted cytokines in both cell lines. In contrast to H4 cells, meningitic *C. sakazakii* strain 767 suppressed the expression of TLR7 in Caco-2 cells, while *C. sakazakii* 709 significantly upregulated this gene more than 4000-fold compared to untreated cells, and as mentioned previously this extreme expression did not correlate with excessive cytokine expression. This indicates that the expressed mRNA may not be translated to active receptor, and that the changes in this gene expression in H4 cells is more aligned to the pathogenic history of the investigated strains.

Human cells have developed a comprehensive system to detect an extensive variety of PAMPs and damaged associated molecular patterns (DAMPs) such as bacterial DNA and cell wall components as well as host self-components including host RNA and DNA (Marshak-Rothstein and Rifkin, 2007). TLR8 is also expressed intracellularly on the membranes of vesicles, endosomes and lysosomes and can detect bacterial transfer RNAs (tRNA) and Gram-negative RNAs (Gehrig *et al,* 2012; Jöckel *et al,* 2012; Cervantes *et al,* 2013). Active protein of these TLRs is expected to recognize both PAMPs and DAMPs of pathogens able to invade human cells, and the downregulation of the expression of this gene hypothetically will increase the ability of these strains to escape the immune system. However, TLR8 was induced in both cell lines by most of the same bacterial strains that enhanced TLR7 expression, especially those associated with fatal meningitis or septicaemia. Similarly, it is possible that the transcribed mRNA of this receptor was not translated to active protein or that the experiment duration was not long enough to finish this process.

Human Embryonic Kidney 293 (HEK) cells transfected with TLR7 and TLR8 display an increased expression of TLR7 and TLR8 in response to bacterial RNA from *E. coli* (Karikó *et al,* 2005). Moreover, different studies indicate that bacterial nucleic acids can induce TLR8 activation (Cervantes *et al,* 2013) in different cell lines, such as THP-1 cells, following phagocytosis of *Mycobacterium bovis* (Davila *et al,* 2008), and *Helicobacter pylori* (Gantier *et al,* 2010). Strains that induced high TLR8 expression, further exhibited different phenotypic characteristics including invasion, translocation over polarized monolayer and cytokine induction in both H4 and Caco-2 cell lines, indicating that the expression of this gene may be activated internally throughout a specific signal pathway, which requires further investigation. Although Caco-2 cells demonstrated higher gene expression than H4 cells in response to certain strains (compared to untreated cells), the expression in H4 cells was more associated with clinically significant strains. TLR8 acts with TLR2 to induce production of NF-kb-dependent cytokines that play a key role in host immunity and pathogen clearance (Cervantes *et al,* 2013) and therefore the increased expression if these receptors were translated to active receptor proteinswould be expected to increase the cell's sensitivity to pathogens and increase its immune response.

TLR9 is generally found in vesicle membranes and is thought to be involved in control of *Mycobacterium tuberculosis* infection through recognition of bacterial DNA (Chen *et al,* 2010). Pedersen *et al,* (2005) showed that the expression of TLR9 is in response to CpG-oligonucleotides was not detected in Caco-2 in contrast to HT-29 cells as shown by Western blotting of the cell lysates, which is in contrast with the results of our study where some bacterial strains upregulated the expression of this gene in Caco-2 cells more than 900-fold compared to untreated cells. These results suggest that TLR9 expression differ in response to bacterial species and possibly bacterial strains. In contrast to Caco-2 cells, most strains suppressed the expression of this gene in H4 cells, and only septicaemic isolate *C. sakazakii* ST4 and *C. turicensis* strain 1211 did not affect the expression. However, among the *Cronobacter* isolates, *C. sakazakii* ST4 strain 709 upregulated most of human TLRs, compared with other isolates including those form the same ST. Therefore, further studies are needed to understand the mechanisms used by this strain to dysregulate the expression of these genes that are not used by other *Cronobacter* strains, including members from the same ST. Interestingly, this strain was

also the strongest inducer of IL-1β secretion, a key activator for host immune response, which might be linked by another specific pathway that in addition, needs more clarification.

TLR10 is described as a vital inhibitory receptor and blocking of this TLR results in increased TLR2-medited cytokine production including TNF-α, and IL-6 upon activation of cells with ligands of TLR2 (Oosting *et al,* 2014). In the present study, TLR10 expression was downregulated by most strains in H4 cells. This downregulation was associated with high inflammatory cytokine secretion, which is in broad agreement with the findings of Oosting *et al,* (2014). However, this increased inflammation may be because of the limitation in the active protein production. *C. sakazakii* ST4 strain 709 did not affect the expression of this gene.

Caco-2 cells displayed increased TLR10 expression and produced lower levels of cytokines, which supports the findings of Oosting *et al,* (2014). However, due to the weak correlation between results obtained using Caco-2 and pathology linked to most strains, this cell line may not be the best model to investigate neonatal inflammatory response to bacterial infection. However, our findings suggest that TLR10 may play an important role in the regulation of host inflammatory response, and results obtained using H4 cells were in closer agreement with those of previous studies (Oosting *et al,* 2014) and the association with pathology associated with particular strains and it might be a better model of bacterial enhanced inflammation compared with Caco-2 cells.

NF-kB is a key regulator of mucosal immunity and modulates the secretion of pro-inflammatory cytokines and chemokines as well as the apoptotic mechanisms in intestinal epithelial cells (Jobin and Sartor 2000), and is expressed by almost all cell types in response to a significant variety of external stimulants (Oeckinghaus and Ghosh, 2009). However, dysregulation of NF-kB has been involved in several diseases because of its ability to influence expression of different genes, thus, the activity of this NF-kB is firmly controlled at different levels (Oeckinghaus and Ghosh, 2009). Therefore, the effect of bacterial challenge on the expression of two members of this regulatory factor subunit1 (NF-kB-S1) and subunit2 (NF-kB-S2), known as p50 and p52, respectively, was examined (Christian *et al,* 2016).

The effect of bacterial strains onexpression of both subunits significantly varied and important differences between the two cell lines were observed. Most of the strains increased the expression of NF-kB-S1 except *C. sakazakii* strain 709 and *C. turicensis* strain 1211, which displayed no effect. This upregulation coincided with an increase in the inflammatory cytokine production by most strains.

With regard to NF-kB-S2, the minor increase in the level of expression that was recorded for two of the most clinically important strains did not associate with specific p[roduction of specific virulence factors or phenotype as most of the strains that disrupted gene expression were able to induce high cytokine production. *E. coli* K1 strain showed the maximum suppression of NF-kB-S2, which is completely in contrast with its effect on NF-kB-S1. Most of the other strains tested showed no effect on the expression of the NF-kB-S1 in Caco-2 cells and increased the expression of NF-kB-S2, which is in contrast with results obtained for H4 cells. The increase in expression of NF-kB-S1 in H4 correlated with the high levels of produced cytokines, with no link with NF-kB-S2 expression. This suggests that expressed mRNA of NF-kB-S1 in H4 was processed to active protein and is involved in the activation of host immune system. In contrast, there is a stability in expression of subunit1 in Caco-2 cells which did not affect cytokine production. However, the increase in expression of subunit 2 did not affect the production of cytokine, which strongly supports our hypothesis that subunit 1 is the key regulator of host immune response and cytokine production.

Berin *et al,* (2002) found that Enterohaemorrhagic *Escherichia coli* O157:H7 (EHEC) can induce NF-kB with a remarkable increase in IL-8 production in different cell lines including Caco-2 cells. In their study the induction of NF-kB expression and IL-8 production was most likely flagellin-dependent, and not induced by other virulence factors such as shiga toxin. This suggestion was confirmed using purified flagellin (Berin *et al,* 2002). However, in their study they did not determine whether the increase was in subunit1 or 2, or even one of the other family members.

In general, the increase in gene expression may indicate an increase in the number of the receptors, which may amplify the corresponding ligand detection by host cells and activation of immune system. Strains downregulating specific TLRs could escape host immune responses and a gain better chance of survival and proliferation. However, this

possibly was not verified by our study as most of the strains that suppressed these genes induced high pro-inflammatory cytokine production.

Results shown in this chapter further support our suggestion that H4 results are more relevant to both clinical history and inflammatory response results, and it would be a better model in host pathogen interaction to investigate neonatal infection and disease development. Moreover, inflammation is a key factor in disease development and impropriate activation of the host immune system can lead to serious complications including facilitating bacterial invasion and translocation to deeper tissues or even long term immune diseases. Thus, we strongly propose use of H4 cells, instead of Caco-2 and other adult derived cell lines, in investigating neonatal bacterial infection and host responses. However, although we show in this study that H4 is more responsive and displayed more sensitivity to bacterial attack, the underlying mechanisms are still not completely understood. We could determine that the expression of NF-kB1 is more inducible and associated with the increased inflammatory response in neonatal cells, while the changes in NF-kB2 did not link to the increased response. Moreover, there was a slight upregulation in NF-kB2 in Caco-2 cells which did not show any link with cytokine production, whereas a potential link was observed with downregulation of NF-kB1, and low cytokine production. Therefore, further investigation is needed to better understand the infectious mechanisms employed by neonatal cells in response to the emergent pathogen *Cronobacter* and other pathogenic microorganisms, to help safeguard the precious lives of new-borns. Only *C. sakazakii* 709 slightly upregulated the expression of TLR1-4, and markedaly upregulated the expression of most of other TLRs in both cell lines, which was not observed by other ST members such as 701 and 767. Therefore, further investigations at the molecular and genetic levels to explain these differnces is needed.

In conclusion, the production of inflammatory mediator s was mostly higher by H4 cells despite the downregulation of TLRs by most strains, suggesting that H4 cells are more inducible to develop inflammation. To support this suggestion, we compared the expression of TLRs in both cell lines with the house keeping gene β-actin in non-treated cells and we found that TLRs 5-8 were expressed 2-4 q-PCR thermal cycles before β-actin which is unexpected (Figure 5-16).

However, the human inflammatory response is regulated by different mechanisms and different signal pathways are involved, and the PRRs are composed of different sensors including TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoid acid-inducible gene I (RIG-I)- like receptors (RLRs), and others (Fukata and Arditi, 2013). All of these receptors participate in the recognition of foreign pathogens, and play a partial role in activation of immune system and fighting disease. Thus, the mismatching of the unbalanced regulation of the investigated TLRs and detected inflammatory cytokines and other factors could be due to one of these PRRs is possible that this dysregulation of TLRs affects unknown mechanisms that lead to this exaggerated inflammation observed in neonates and in the recent study by H4 cell line. Finally, we looked at gene expression which is not the final production of receptors, which still need additional processing and modification to become active protein. In addition, as we used non-polarised cells, it is not possible to decide which aurface (basolateral or apical) of host cells are more sensitive to stimulation and how this may related to increased inflammation. This requires further investigation using polarised mono/bilayer neonatal and adult cell lines.

Chapter 6. **Conclusion.**

Neonates are exposed to bacterial infection with mortality rates much higher than that recorded for adults. Intensive research has been focused on the role of microbial infection in neonatal immunity and activation of their immune responses. Bacterial infection is considered one of the major causes of neonatal deaths with about 3 million deaths annually. Gram-negative bacteria, and particular the Enterobacteriaceae are involved in neonatal infection worldwide with reported high incidence of case fatality. Among this family, the genus *Cronobacter* have been recently shown to be involved in severe neonatal infections including meningitis, septicaemia and necrotizing enterocolitis. Neonatal infections with *Cronobacter* has been largely associated with the consumption of contaminated powder infant formula (PIF). This genus was involved in globally distributed outbreaks in neonatal intensive care units with high morbidity and mortality rates. However, most of published data are based on results obtained from host–pathogen interactions where the research was performed using non-neonatal cell lines.

Thus, in this thesis, the H4 non-malignant neonatal intestinal epithelial cell line was introduced as a new model for the study of host-pathogen interactions. We aimed to determine key virulence factors of some pathogenic bacterial isolates, in an attempt to discover the mechanisms behind the high susceptibility of neonates, compared with adults, to bacterial infection. *Cronobacter* genus was selected due to the global interest in this genus, since it was involved in serious illnesses and outbreaks in the last few decades with reported fatal cases especially for *C. sakazakii* species.

The present study was designed based on the following aims:

- Investigation of the phenotypic diversity of selected *Cronobacter* strains including the seven type species strains and selected clinical isolates associated with infections in neonates and young children. We aimed to determine whether the differences between

these strains affected their virulence when co-cultured with human cell lines and if particular phenotypes can be associated with certain pathologies.

- Investigation of the ability of the selected strains to attach, invade, translocate and induce cytotoxic effects on human cells, and the role of the human cytoskeleton in this process. In addition, the production of highly cytotoxic mediators that might facilitate bacterial infection was investigated.
- Characterization of the host response through quantification of a wide range of cytokines involved in the activation of the host immune system.
- Assessment of the role of human TLRs in the host immunity and evaluation of TLRs potentially involved in this process, through analyses of gene expression during bacterial challenge.

6.1 Bacterial physiological traits

In order to evaluate the effect of growth rate on the attachment and invasion studies, the growth profiles in TSB and tissue culture medium were performed, as few host-pathogen studies evaluate the differences in bacterial growth rate in tissue culture medium. In general, although some strains showing high growth rates and in addetion were among the most highly capsulated, most motile and highest biofilm producers, that bossibly indectae to the correlation between the traits, for other strains the correlation between growth rate results and other phenotypical characters is not clear. For example, *C. sakazakii* strain 513 exhibited high growth rates, but was non-motile and moderate-low biofilm producer.

Moreover, the growth rates did not correlate with some of the other investigated virulence factors, as we expected, such as bacterial adherence and activation of cytokine production. This indicates that for bacterial tested in this study, unless they possess a key factor to facilitate the adherence and induce inflammation, an increase in bacterial numbers was not a major factor in invasion or cytokine production. However, despite

the weak overall correlation of growth rates and other virulence factors in this study, bacterial cell numbers increased at different rates. This should be taken into account when looking at their interaction with human cells in tissue culture as it may still have an important role for some pathogenic strains.

Bacterial motility is involved in different virulence traits such as biofilm formation, and may contribute to bacterial adhesion and invasion in mammalian cells. Bacterial flagella can trigger host cells to exacerbate the inflammatory response leading totissue damage thereby facilitating bacterial spread into deeper tissues. Generally, highly motile strains such as *C. sakazakii* stains 701 and 767, and *C. malonaticus* strain 1569 were among the high biofilm producers especially on enteral feeding tubes, and showed high adherence to human cells and IL-8 induction. Non-motile *C. sakazakii* ST8 strain 513 exhibited weaker cytokine induction in H4 cells, with no evident link between motility and presence/absence of motility-related genes. *C. sakazakii* ST8 strain 513 harbours *fli* and *flg* genes as well as the *pilT* gene which is linked to twitching motility, but was non-motile. Further investigation tostudy the expression of these genes is required, and microscopic investigation for motility organelles will help in better understandin of thee mechanesms.

Strains were investigated regarding biofilm production in different types of milk and abiotic surfaces. Most clinical strains such as the meningitic *C. sakazakii* 767 and *C. malonaticus* 1569, as well as other ST4 isolates showed significantly higher biofilm formation in C&G milk, a whey-based milk compared to SMA, a casein-based product, in 24-well plates and on EFT milk ($P<0.0001$/paired t test). However, strains with less clinical importance showed no significant differences between C&G milk and SMA milk on the enteral feeding tubes. Biofilm formation is more likely to depend on the surface and milk ingredients rather than individual differences between individual strains. No link was found between presence/absence of the genes potentially involved in biofilm formation, which may be explained by lack of expression or regulation by other genes.

Capsule production results indicated that the most of bacterial strains tested could produce capsules to a greater extent when grown in milk than on other types of substrates such as XLD and VRBGA. Capsule type can be diverse from one strain to

another and certain capsule types can cause serious neonatal infection (i.e. K2:CA2: Cell+). This capsule type is common among *C. sakazakii* ST3*,* clonal complex 4 (CC4), and ST12 and is also found in different species and STs such as the fatal meningitis isolates *C. sakazakii* ST31 strain 1249 and *C. malonaticus* ST307 strain 1569 (Ogrodzki and Forsythe, 2015). Almost all significant *C. sakazakii* strains were K2:CA2: Cell+ capsule type. Despite the association of K2:CA2: Cell+ capsule type with severe infections, there was no obvious link with the presence or absence of capsule-linked genes, suggesting that the nutrients available play a crucial role in capsule production.

Congo red binding is thought to indicate production of curli fimbria and exhibits a red phenotype on the Congo red culture medium (Yan *et al,* 2015). Strains exhibiting red colour largely had important clinical histories such as *C. sakazakii* TS4 strain 701, which was originally isolated from peritoneal fluid of a neonate with fatal NECIII (died), and *C. malonaticus* 1569, a neonate fatal meningitic isolate. However, Congo red also binds to cellulose polymers expressed by some pathogenic bacteria such as *E. coli*, which may result in miss-identification as Congo red-positive. This possibly occurs in *Cronobacter* isolates (Hung *et al,* 2014) and may explain the absence of the correlation between Congo red binding and adhesion or invasion of some strains.

Blood cell lysis by certain bacterial isolates could indicate the production of cytotoxic factors that can destroy cell membranes. This mechanism may be utilized by pathogenic bacteria to contribute to necrotic damage in the site of infection and the development of infection. Most *Cronobacter* strains exhibited β-haemolytic activity on horse blood. Presence or absence of haemolysin-associated genes such as *hlya* (Dhakal and Mulvey, 2012) and *ykfi* (Wen *et al,* 2017), as well as the ST seem to not have a clear role in this activity. However, strains with less significant pathology were non-haemolytic, suggesting a role of blood haemolysis activity in disease occurrence.

Gastric acid is considered one of the obstacles against bacterial survival and growth. A previous study indicated that neonatal gastric acid could go down to as low as pH of 1.5 (Smith, 2003; de Jesus *et al,* 2005; Hurrell *et al,* 2009 and Alvarez-Ordóñez *et al,* 2014), therefore, strains were exposed to pH 2 for 2hrs. None of tested strains was recovered after the incubation period, suggesting that *Cronobacter* strains may employ different

strategy to avoid this extreme acidic environment in the stomach of neonates. However, different studies have investigated the sensitivity if *Cronobacter* spp to acid stress. Edelson-Mammel et al (2006) showed that the exposure of 12 *Cronobacter* isolates to pH of 3 and 3 .5 resulted in decline of bacterial survival by 1 and between 5 and 6.3 log cycles respectively. This is supports our findings as no growth recovery was obtained after 2h at pH 2. Notably, this assay was performed in TSB, which may provide less protection to the pathogenic bacteria compared with milk. Thus, further investigation using different milk types is required, as this project provided evidence on the role of milk in bacterial biofilm production, an important virulence factor in bacterial survival.

6.2 Host-pathogen interaction

This study aimed to investigate the ability of different *Cronobacter* isolates and one *E. coli* K1 isolate to attach, invade and translocate over polarised monolayers of the neonate-derived cell line H4, and compare the results with the adult-derived Caco-2 cells. Moreover, the cytotoxicity of the selected isolates was investigated in order to evaluate whether they are able to produce cytotoxic compounds to overcome host cell resistance. In addition, the role of human cytoskeleton in bacterial invasion was also investigated. After bacteria succeed to invade and reach the blood stream, it has to survive the bactericidal components in blood to be able to cause disease. Therefore, the serum resistance assay was also performed for the investigated strains.

Bacterial adherence clearly differed between the two cell lines, which is significantly higher in H4 cells compared by Caco-2. However, strains associated with important pathology demonstrated high levels of attachment to both cell lines. Results in H4 cells displayed better correlation with the clinical history of investigated strains, most of the clinically important strains were in the top 10 adhesive strains to H4 cells compared with only one strain among the top 10 for adhesive to Caco-2 cells (Figure 4.3 and 4.5). Significant differences were obtained between the attachment of NEC isolates and strains which were not associated with serious disease, ($p < 0.0001$/ Ordinary one-way ANOVA).

The level of adhesion was linked to the growth rate of some bacterial isolates, but no clear link between motility and adhesion was evidenced for non-motile strain 581 that displayed high level of adhesion. Most strains were more adhesive to H4 cells than Caco-2 cells, suggesting the potential link between bacterial attachment and the excessive inflammatory response in neonates.

Moreover, *Cronobacter* strains invaded both H4 and Caco-2 cell lines. Clinically important strains were among the most invasive for H4 cells. *C. sakazakii* ST4 isolates showed high levels of invasion in both cell lines compared with that of non-ST4 isolates. Two of these strains, 767 and 701, were responsible for neonatal deaths due to meningitis and NECIII, respectively, which is in agreement with the association of this sequence type with neonatal meningitis, proposed before (Joseph and Forsythe 2012c, Forsythe et al, 2014). In general, *C. malonaticus* isolates were more invasive in Caco-2 while *C. sakazakii* were more invasive in H4 cells.

In addition, all of the tested strains were able to translocate through polarized monolayers of human cells causing necrotic damage in the polarized human cells, quantified as a drop in the TEER of the infected cells which was largely higher in H4 cells. Bacterial isolates associated with blood infection or meningitis were the highest translocated strains. *C. sakazakii* ST4 701 (NECIII isolate) and 709 (septicaemic isolate) from French outbreak translocated through polarized H4 cells more than Caco-2 cells.

The number of translocated bacteria was higher than for the invasion and several reasons could be involved, including the infiltration of pathogenic bacteria between epithelial cells, and through the damaged spots in the polarised layer rather than invasion. Another possible reason is that during the invasion assay gentamicin kills the bacteria outside the cells, and only invasive bacteria are counted, while in the translocation assay, bacteria have a chance to replicate in the basolateral chamber.

Serum resistance is a very important virulence factor for pathogenic bacteria that succeed in reaching the blood stream. Interestingly, the top 11 resistant strains were *C. sakazakii* isolates that showed significant resistance compared with the negative control and some other *Cronobacter* strains. Significant resistance was also recorded for *E. coli* K1 strain 939. Blood isolates such as *C. sakazakii* strain 1249, the fatal meningitic isolate,

and strain 709, a neonatal severe septicaemia isolate, were able to replicate in the serum and displayed significant increases in cell numbers after 2hr incubation. The ability of these strains to survive in serum may explain their resistance to the host immune system, which allowed them to survive in the blood and infect different host organs such as brain and meninges. The meningitic *C. sakazakii* 1249 was among the highest translocated strains, and together with its ability to survive serum complement, may explain whythis strain can cause serious infection in neonates.

Genes responsible for serum resistance were assessed in the investigated strains, but no clear association was found between virulence and genes such as *cpa* apart from a possible link between the high resistance demonstrated by *C. sakazakii* strains and the presence of hypothetical protein ESA_04103 described by Schwizer *et al,* (2013). However, bacterial LPS known to play important role in serum resistance and shortness of the O-antigen side chain of capsular polysaccharides associate LPS was linked to loss of serum resistance (McCallum *et al.,* 1989; Doorduijn *et al.,* 2016). Merino *et al,* (1992) indicated that adding of purified LPS from *K. pneumoniae* to serum sensitive starains was effectively reduced serum-mediated killing.

Host cytoskeletal components such as microfilaments (MF) and microtubules (MT) were described to be used by pathogenic bacteria to enhance their invasion of host cells. Invasion of H4 cells was significantly reduced with MF inhibitor Cytochalasin D to 60-80% for *Cronobacter* isolates and 50% for *E. coli* K1, whereas MT inhibitor Colchicine reduced the invasion up to 98% for *C. sakazakii* strain 701. In contrast, Nocodazole increased the invasion of some strains to H4 cells and slightly effected the others. Conversely, Cytochalasin D enhanced the invasion to Caco-2 up to fivefold of the controls.

Generally, invasion of clinically important strains such as NECIII and meningitic isolates from *C. sakazakii* and *C. malonaticus* in H4 cells was significantly reduced upon inhibition of both MF and MT by Cytochalasin D and colchicine, respectively ($P < 0.0001$/ Chi-seqare test), suggesting that these strains are able to utilize the neonatal cell cytoskeleton to facilitate invasion. The invasion of those strains importantly increased in Caco-2 cells treated with these inhibitors, indicating a different role for human cytoskeleton in the control of bacterial invasion in adults. In contrast, pathogenic bacteria may exploit the

immaturity of neonatal intestinal cells and utilize their cell membrane-associated cytoskeleton to contribute to infection and disease. Therefore, this suggests that H4 cells represent a better model to mimic the pathology associated with clinical isolates in neonates.

Moreover, data obtained in this experiment suggests a possible strain-specific role for these inhibitors, that the investigated bacteria encode different pathways for their uptake, and differnces specific dependant on the eukaryotic receptors that recognize the invading bacteria.

Cytotoxicity of pathogenic bacteria can damage host tissues and facilitate translocation to deeper tissues. Most of the bacterial strains showed ability to kill human cells, and H4 cell was mostly more sensitive to bacterial cytotoxicity. This assay allowed us to discover the extremely cytotoxic *C. sakazakii* ST3 strain 978, originally an enteral feeding tube isolate. This strain showed cytotoxicity for H4 cells which was 90-fold higher than that detected untreated control H cells and was approximately 15-fold higher than citotocic activities showed by compared with maximum six-fold shown by *C. condimenti*. With Caco-2 cells this strain showed cytotoxic activity wich was 0-fold compared to untreated cells. High cytotoxicity was also observed *for E. coli* K1 strain 939. Interestingly, selected ST3 isolates displayed low cytotoxicity to both cell lines. However, only the two strains 978 and 984 were isolated from clinical sites (EFTs), while all the available ST3 strains are food or environment isolates.

The 978 strain was further investigated to determine the potential reason of this excessive cytotoxicity (see section 4.3.5.6). Findings indicated that only live bacteria can cause this level of cytotoxicity. In addition, this strain must be able to produce new proteins as confirmed by chloramphenicol assay. Moreover, bacteria have to be physically in contact with host cells, which was evidenced by incubation of the bacteria in 0.4 µm inserts instead of directly with host cells. This finding suggests the possible involvement of a secretion system (SS) in generation of cytotoxic effects. Genetican alysis study indicated this strain harbour 16 genes encoding four different types of SS (TSSs) including I, II, IV and VI. Different studies indicated that T2SS, T3SS and T6SS can contribute to bacterial pathogenicity by increasing the toxic effects on host cells (Suarez

et al., 2008; Bleumink-Pluym *et al*, 2013). However, according to our finding, the toxic protein most be injected into the cells, as no effects was observed when bacteir were separated from host cells bt inserts, and then injected proteins possibly work at the physiological level or DNA and enhance programmed cell death.

6.3 Host response and gene expression.

All strains were able to induce IL-8 induction, and levels displayed by *C. sakazakii* strains and the other fatal *Cronobacter* isolates were the highest mainly in H4 cells. With regard to the amount of secreted IL-8, H4 cells showed higher response than with Caco-2 cells.

In general, *C. sakazakii* strains were more stimulatory on H4 cells than other isolates, while there was a high variation in Caco-2 response to these strains. Non-motile *C. sakazakii* ST8 strain 513 was the lowest IL-8 inducer in H4 cells, whereas non-motile *C. universalis* strain 581 was among the high IL-8 inducers, while no differences on the effect of the ST8 isolates were observed in Caco-2.

These results support our hypothesis that H4 cells represent a better model of host response, and reproduce the excessive inflammation observed in neonates upon bacterial infection, which aligns with previous findings indicating that H4 cells are more inducible by bacterial infection, despite the limited bacterial isolates used in those studies (Erika *et al,* 2003; Savidge *et al,* 2006).

Bacterial OMPs from different *Cronobacter* isolates and from *E. coli* K1 strain 939 were able to induce IL-8 production in both cell lines. Interestingly, stimulation by OMPs exhibited a pattern similar to that indicated by the corresponding live bacteria in both cell lines. OMPs of *C. sakazakii* ST4 strains 701, 767 and 709 were among the stronger IL-8 inducers, while the OMPs of *E. coli* was among the weaker IL-8 stimulants, matching the ELISA results obtained when live bacteria were used. In general, H4 cells were more inducible by bacterial OMPs than Caco-2 cells ($P<0.0001$/ paired t test). However, we did not examine whether the extracted OMPs are contaminated with bacterial LPS, future work could investigate the effect of *Cronobacter* using purified OMPs and LPS separately on the neonatal H4 cell line.

IL-8 production by polarized monolayer epithelial cells indicate that both cell lines exhibit a bidirectional inflammatory response dependent on the site of infection (See section 5.3.2-5 for the results). IL-8 secreted from the basolateral site after one hour incubation was much lower than the upper site, and there was an increase in the amount of IL-8 in the basolateral side with time. This increase could be either in response to apical or basolateral stimulation by the translocated bacterial cells. Some strains that showed low translocation through the polarized monolayer, such as *C. sakazakii* ST4 strains 701 and 709, induced greater IL-8 secretion at the basolateral side more than the upper side (see figure 4.9 for translocation and figure 5.4 for IL-8 induction). In Caco-2 cells, IL-8 secretion depended on the site of stimulation and the concentration of recovered IL-8 was much higher in the upper side cells stimulated by bacteria (see figure 5.6).

In conclusion, H4 cells were more responsive to bacterial challenge than Caco-2 cells and secreted higher levels of IL-8 in response to stimulation with live bacteria and their OMPs. However, in terms of clinical history and disease caused by these strains, only three clinically significant strains were among the top ten IL-8 inducers in H4 cells, compared with six clinically important strains among the top 10 producers in Caco-2. This observation should be taken into the consideration for future work to gain better understanding of the differences between these two cell lines, and more research is vital to determine the appropriate model for investigation of neonatal infection and related inflammatory diseases.

Human cell lines were further investigated for the secretion of different cytokines and growth factors using Magnetic bead-based assays on the Luminex platform and results were varied.

IL-1β: All of bacterial strains enhanced IL-1β secretion by H4 cells with exception of *C. sakazakii* ST8 isolates strains 1 and 513. This might be because this sequence type is not one of IL-1r ligands or these strains blocked IL-1 receptors through an unknown mechanism. However, only one meningitic *C. sakazakii* strain 1249 was able to induce IL-1β by Caco-2 cells.

IL-6: H4 cells secreted IL-6 up to 20-times higher than Caco-2 cells in response to some bacterial isolates (Figure 5-8). The highest concentration of IL-6 produced by H4 cells was in response to *C. sakazakii* 1557, the most invasive in H4 cells, suggesting that intracellular receptors could induce IL-6 production. Comparatively, very low IL-6 was produced by Caco-2 cells, and there was no correlation with adhesion and invasion. However, *E. coli* K1 939 strain 939 was the second IL-6 inducer in H4 despite the low invasion but very effective attachment to H4 cells.

IL-8: in agreement with previous examination with ELISA, there were major differences between the two cell lines and H4 was more inducible by bacterial infection. The concentrations of IL-8 detected by this method were significantly higher than detected using normal ELISA, and the highest IL-8 production was in response to *C. sakazakii* strain 1557 matching the results for IL-6. Non-motile ST8 strain 513 was the weakest stimulant among *C. sakazakii* isolates although this strain was more adhesive than the ST8 *C. sakazakii* strain 1. Similar to normal ELISA, *C. sakazakii* was the strongest IL-8 inducer. In contrast to traditional ELISA method, *E. coli* K1 was among the stronger stimulants of cytokine production in H4 cells. This was unexpected and it could be due to the experimental conditions or because of the high sensitivity of this kit compared to normal ELISA.

Monocyte chemoattractant protein 1 (MCP-1): Both cell lines produced detectable amounts of MCP-1, except *C. sakazakii* ST8 strains that did not induce Caco-2 to produce detectable amounts of this cytokine which is very important in triggering immune cells and helps in bacterial clearance. No relationship was observed between MCP-1 production and other virulence factors such as attachment and invasion, with the only possible link between motility and MCP-1 shown in non-motile *C. sakazakii* ST8 strain 513 and motile strain 1.

Interferon gamma (IFN)-g induced protein 10 (IP-10): Both cell lines produced small amounts of this chemokine which can be used as a positive marker in early stages of microbial infection. *E. coli* K1 strain was the strongest stimulant for H4 cells, while *C. malonaticus* 1569 was the strongest inducer for Caco-2 cells. High concentrations of this protein have been linked to NEC infection in human neonates (Ng *et al, 2007),* and

although the detected amounts were very low, H4 exhibited more IP-10 secretion than Caco-2 cells which better mimics the neonatal vulnerability to NEC than seen in adults.

IL-10: IL-10 is an important anti-inflammatory cytokine that plays an important role in regulating pro-inflammatory cytokine production (Ouyang *et al,* 2011). The results obtained are largely in agreement with previous studies suggesting that IL-10 is not expressed in epithelial cells (Haller, 2000; Vinderola *et al,* 2005), although it was reported that IL-10 was detected at mRNA level in intestinal epithelial cells (Bahrami *et al,* 2010), but that the translation to active protein may not have occurred.

IL-4: there was a variation in the response to bacterial strains, and non-motile *C. sakazakii* ST8 strain 513 was the weakest inducer for H4 cells. Caco-2 cells were nonresponsive to about 38% of bacterial isolates compared to 7% in H4 cells. Although this cytokine is known to regulate the host inflammatory response, *C. sakazakii* strain 701 and *E. coli* K1 were among the stronger stimulants for this cytokine, and were also among the strongest inducers for the inflammatory cytokines such as IL-8 and IL-6. This suggests that the secretion of these cytokines by H4 cells is regulated by different mechanisms, and the expression of IL-4 at the detected levels was unable to downregulate the inflammatory cytokines secretion by this cell line.

EGF: All strains showed the ability to enhance EGF production in H4 cells, while most of bacterial isolates did not induce the secretion of this growth factor by Caco-2 cells. Only clinically significant strains induced low EGF secretion by Caco-2 cells (2.7 pg/ml-8.6 pg/ml). EGF was not detected in both untreated cell lines. This growth factor is important for mucosal development and repair, and was expected to be suppressed by some strains such as *C. sakazakii* strain 701, the NECIII isolate, but this strain was found among the strong inducers. This suggests the potential role of long term inflammation in the NEC due to the excessive migration of immune cells such as macrophages to the site of infection, which may lead to auto-immune diseases and induction of mucosal damage.

In addition to the cytokines described previously, H4 and Caco-2 were also investigated in regard to the production of IL-2, IL-12, IFN-γ and TNF-α in response to bacterial infection.

IFN-γ levels were lower than the detection level of this assay (<0.5 pg/ml). IL-12 was secreted by H4 cell line in response to only four strains, two of them clinically significant strains which are *C. sakazakii* strain 709 (septicaemia) and *C. turicensis* 1211 (meningitis).

Generally, these results provide evidence for the differences between adult and neonate cells, confirm that H4 cells respond similarly to previous reports of induced inflammatory diseases and increased autoimmunity due to bacterial infection (Sanderson and Walker, 1995; Claud et al, 2003; Liévin-Le Moal; Servina, 2013), and represent a more suitable model to investigate neonatal immune responses. This enhanced response may elucidate the pathophysiology of some chronic illnesses including NEC. The balance between anti and pro-inflammatory responses in the intestinal epithelium and its regulation requires a more extensive investigation. Our data showing that the enhanced reaction of the neonatal cell line support the notion of that the epithelium in neonates and infants plays a significant role, beyond it function as a physical barrier, in mediating the immune responses, and probably compensating for the lack of a mature immune system.

6.3.4 Gene expression.

H4 and Caco-2 cells were investigated with regard to the effects of bacterial infection on the expression of Human Toll-like receptors 1-10 (TLR1-10). TLR1-4 were upregulated by only *C. sakazakii* ST4 strain 709, isolated from neonate with septicaemia, while other strains downregulated these genes. The downregulation of TLR1-4 in H4 cells was not associated with any specific virulence factor or clinical history of the selected strains including the induction of cytokines secretion, suggesting that the inflammatory response of this cell line is multifactorial and different signal pathways are involved in this process. The changes in TLR1-4 expression at the mRNA levels may not be reflected in the respective protein levels, which would be required for the regulation of cytokine expression or immune response in general.

TLR1 was not expressed in Caco-2 while TLR2-4 was upregulated by some strains. Interestingly, *C. sakazakii* ST4 strain 709 induced a significant increase in the TLR2-4 in Caco-2 cells, together with *C. sakazakii* strain 1557. Upregulation or down regulation of

these TLRs did not correlate with any of the investigated virulence factors, and strains from the same ST exhibited different effects on these genes. This suggests a high specificity of bacterial isolates in activating the host immune system, with host receptors responding independently to each bacterial isolate, or its components.

Increased TLR gene expression is expected in parallel with an increase in the number of the receptors, which would increase bacterial detection by host cells and potentially lead to hyper activation of host immunity similar to that observed in neonates. However, the absence of a link between the secretion of cytokines and the downregulation or upregulation TLR2-4 shown by H4 and Caco-2 respectively, suggesting that the activation of immune response of H4 cells might occur via different receptors and regulated by different genes and at different levels and are not only TLR dependant.

TLR5 in H4 was upregulated by some strains linked to meningitic and septicaemic cases from different *Cronobacter* species, as well as by *C. sakazakii* strain 1557 that displayed the highest invasion in H4 cells. *C. sakazakii* strain 709 upregulated the expression of TLR5 in Caco-2 cells up to about 2200-fold, while it almost completely suppressed the expression of this gene in H4 cells. In addition, *C. sakazakii* strain 1557 was among the most highly invasive strains for Caco-2 cells and upregulated TLR5 expression by about 350-fold suggesting that the regulation of this receptor may also occur intracellularly through specific ligands present in invasive strains such as OPMs or lipoproteins that may not be found in the other highly invasive strains.

TLR6 was upregulated in H4 cells by all the meningitic *Cronobacter* isolates, suggesting that these strains might apply a specific strategy to upregulate the expression of this receptor. This is very important finding as the dysregulation of this gene might be a good marker for the detection of infection before disease development. However, to confirm this finding further investigation is needed. Accordingly, the upregulation of this gene was expected to increase the numbers of this receptor in the host cells, and subsequently promote IL-10 production that can regulate the production of other inflammatory cytokines. However, this is completely in contrast with this project findings as no IL-10 was detected by both cell lines and excessive production of inflammatory cytokines by H4 cells was recorded. However, in this PhD project, results

looked at mRNA levels and not at protein levels, which suggests that the expressed mRNA of these TLRs may not be translated to active proteins that can detect the bacteria and induce IL-10, and other cytokine production.

The expression of TLR6 in Caco-2 cells was increased in response to *C. sakazakii* strain 709 and 1557 and upregulated by meningitic *C. malonaticus* strains 1569 and *E. coli* K1 by more than 200% of untreated cells. Results in both cell lines indicate a possible association of TLR6 upregulation and infection with meningitic pathogens.

Likewise, TLR7 and TLR8 were upregulated in H4 cell line by meningitic *C. sakazakii* isolates 767 and 1249 in addition to *C. sakazakii* 1557 and *E. coli* K1. The active proteins of these TLRs are located in endosomal membranes within the host cell and act to detect the invading bacteria or bacterial components such as bacterial RNA, and can activate the immune system to produce inflammatory cytokines. In contrast, the meningitic *C. sakazakii* isolates almost completely supressed TLR7 and TLR8 expression in Caco-2 cells.

Activation of TLR9 requires the uptake of microbes or some of their components as it located on the endosomal membranes (Chen et al, 2010; Moloney et al, 2015), thus the increased expression of this TLR aids in detection of antigens such as CpG oligodeoxynucleotides (CpG ODN) or bacterial DNA which are potent stimulators of immune cell maturation. This receptor was downregulated by most strains, only *C. turicensis* and *C. sakazakii* ST4 strain 709 had no effect on this gene expression. The expression of this receptor was almost completely blocked by *E. coli* K1. Peigane *et al,* (2009) reported that this serotype can evade host immunity, which is one of the key factors behind its ability to reach the central nervous system. The unique transcriptional activation of this TLR and most of other receptors by *C. sakazakii* ST4 strain 709 highlights the need for further investigation to identify differences between this strain and other ST members, especially because these isolates originated from same outbreak and were clustered together in the pulsed field gel electrophoresis (PFGE) profile published by (Caubilla-Barron *et al,* 2007), therefore, SNP analysis could be one of the possible techniques that could help to differentiate between these strains (Masood et al., 2015).

TLR9 was induced in Caco-2 cells by the same strains that upregulated TLR7-8, *C. sakazakii* strain 709 and 1557, *C. malonaticus* strains and *E. coli* K1, whereas *C. sakazakii* meningitic isolate had no effect on the expression of this gene. Therefore, upregulation of this receptor could result in different mechanisms and both externally and internally. Increased expression of this TLRs is expected to increase its active protein synthesis and since it was activated by its ligands, this TLR enhances bacterial clearance from the infected tissues through triggering the immune cells, therefore, the downregulation of this TLR is expected to increase bacterial load in the infected neonates, which was evidenced by previous studies, and shown by neonatal H4 cells in this PhD project. This finding supports our hypothesis that the neonatal H4 cell line is a better model for investigation of the activation of neonatal immune system by bacterial infection and the role of this TLR in this process.

Regarding TLR10, phylogenetic analysis showed that TLR10 is closely related to TLR1 and TLR6 (Chuang and Ulevitch, 2001; Veltkamp *et al,* 2012), and can form heterodimers with TLR1 and TLR2. This gene was suppressed by all strains except *C. sakazakii* ST4 strain 709 that showed no effect on expression of this gene. Conversely, in Caco-2 an excessive upregulation was noted by *C. sakazakii* ST4 strain 709 and other strains such as *C. sakazakii* 1557 and *E. coli* K1.

Results in H4 cells support the previous findings about the regulatory role of this gene, as it is thought to have an anti-inflammatory effect (Oosting *et al,* 2014), and down regulation of this gene in H4 cells will lead to the decrease in the active copies of the protein, and subsequently it could be one of the key factors of the excessive inflammatory response in this cell line. The upregulation of this gene in Caco-2 cells did not result in significant suppression of cytokine production, which indicates that in this context, H4 cells relate better to the possible patho-physiological role of this gene.

The activation of NF-kB subunits 1 and 2 upon co-culture of H4 and Caco-2 cells with bacterial isolates indicate that the activation of cytokine production is subunit 1-dependent. Most strains upregulated subunit 1 but had no effect on the expression of subunit 2 in H4 cells. In contrast, in Caco-2 cells strains did not affect subunit one but increased in subunit 2 expression.

These findings suggest that the upregulation of subunit 1 may be responsible for the excessive increase in cytokine production in H4 cells, which is less affected in Caco-2 cells, whereas the upregulation of subunit 2 expression in Caco-2 cells did not enhance the inflammatory cytokine production at the levels observed in H4 cells. This is an important novel finding, and further investigation is needed to understand how these two subunits are differently regulated by bacterial infection and the role of each subunit in the activation of the immune system in neonates.

The investigation of TLRs produced some important findings that require further investigation to clarify the regulatory mechanisms involved in the expression of these genes, and their relation to the exacerbated inflammatory response observed in neonates. Although some of the upregulated receptors were expected to associate with increase or decrease in the production of inflammatory cytokines, results did not confirm this expectation. Generally, H4 cells responses correlated better with the clinical history of investigated strains and the role of the investigated genes, and support our recommendation to utilise H4 cell line in neonatal infectious disease research.

According to the gene expression results, strains are varied in their mode of action on the activation of host TLRs expression. Some strains are expected to exploit the activation of TLRs expression to increase host inflammatory response which increases the migration of immune cells to the site of infection. This process, in addition to clearing the invaded bacteria, may help to increase infection in different ways. Firstly, some pathogenic bacteria can subvert and exploit host defences such as *Listeria monocytogenes* that can resides in the cytoplasm of macrophages and other host cells (Finlay and McFadden, 2006). Secondly, the increased inflammatory response potentially leads to auto-immune diseases that consecuently may leads to tissue damage and helps bacteria to translocate and infect different tissue. This proposed mechanism is shown in Figure 6-1 as a possible model of some pathogenicity mechanisms of *Cronobacter* isolates. In contrast, some bacterial strains showed less ability to activate host immune system either via increase cells susceptibility to detection of foreign molecules through increasing the TLR copies on the cell surface or even through induction of inflammatory cytokines production. In the present PhD project *C. malonaticus* strain 1546 is good example for such bacteria.

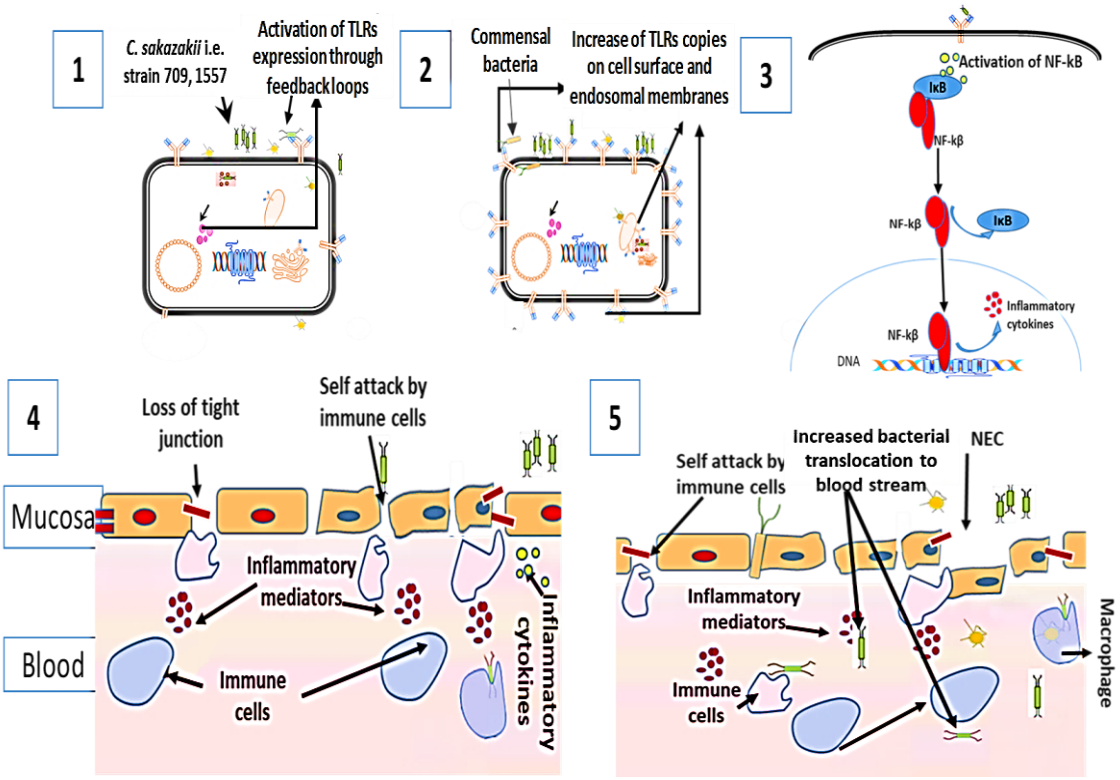


Figure 6-1. The proposed progressive steps of *C. sakazakii*-host interactions and pathogenesis. This steps are expected to be applied by *C. sakazakii* septicaemic strain 709 and variable behaviour of othe *C. sakazakii* strains. **(1)** Detection of attacking bacterial cells through the host cell pattern recognition receptors mainly TLRs (of H4 and Caco-2 cells) that might leads to activation of TLRs expression through feedback loops mechanisms. (**2)** Expressed mRNA possibly will be translated into active protions to take their position on cell membrane such as TLRs 1, 2, 6, 4 and 5 or endosomal membrane like TLRs 3, 7, 8 and 9. This however increases the TLRs copies and subsequently will increase the ability of host cells to detect different pathogens and possibly the commensal bacteria. This detection will increase host immune response and migration of immune cells to the site of infection. **(3)** Activation of NF-kB, the important activator for inflammatory cytokines expression**. (4)** Exaggerated immune response might lead to self-attack and loss of tight junction. **(5)** Prolonged inflammation can possible lead to NEC occurrence and diffusion pathogenic bacteria to blood stream causing septic shock and/or infection of different tissues such as brain and meninges causing meningitis.

6.3.5 Future work.

To confirm this project's findings, further studies are required. The studies of genus *Cronobacter* with the H4 cell line suggest mecjhanim by which this bacterium may possibly be involved in neonatal meningitis and NEC. For better understanding it is may be useful to use different neonatal cell lines especial non-transformed cell lines to better mimick the real life situation. Moreover, host-pathogen interaction experiments that are based on monolayer studies, may limit the interactions between the host cells during the infection, therefore, invasion and translocation of polarised bilayers is vitally important to support the in vivo/in vitro findings. In the recent project we found that different *Cronobacter* isolates were able to cause necrotic damage in the polarized monolayer, as confirmed by losing of the TEER. This would be better understood if it was confirmed with neonatal intestinal tissues, in which immune response studies will help in better understanding the mechanisms of NEC. However, bacterial flagella are thought to activate cytokine production by human cells, and as this was not completely clear in this project, especially with *C. universals* 581 which is non-motile strain but still induced inflammatory response by both cell line. While the non-motile *C. sakazakii* 513 did not. Structural studei using electron microscopy can confirm if both strains have flagella or not, then motility assay could be used to investigate if *C. universals* has inactive flagella while *C. sakazakii* 513 does not. However, differences seen in this project between these to strains may also associated to other factors unrelated to flagella. However, as some of Cronobacter isolates showed important link between motility and cytokines production such as *C. sakazakii* 513, while other strains from different species showed no link. This could be an initial work for collection of non-motile Cronobacter isolates and investigate them in regard the activation of immune system.

Failure to control immune responses against microbiota, environmental or self-antigens can raise the chances of several illness such such as allergies, inflammatory disorders and autoimmune condition (Belkaid and Hand, 2014). Therefore, intensive research into the mechanisms of increased inflammatory response in neonates compared to adults is required. This project provided clear evidence about the differences of the effect of particular bacterial strains on the cytokine production between adult Caco-2 cells and

neonatal H4 cells. Quantification of cytokines and growth factors using the magnetic beads is also needs to be repeated as there were significant differences (P<.001/paired t test) in produced IL-8 between this and ELIA based methods, although the same samples were used in both assays.

For better understanding of effects on cytokine production it would be useful to conduct gene expression assays for these cytokines and compare results between the two cell lines. It is possible that not all expressed mRNA of these inflammatory mediators is translated to active proteions. Results obtained could then be compared with ELISA results which will help to understand if the different cell line control inflammatory cytokine production differently, even after they have been expressed as mRNA.

TLRs group are PRRs and play a crucial role in controlling infection and activation of the immune system. Our finding indicated to the specific role of single bacterial isolate with each cell line, which requires further investigation using different neonatal cell lines, and to continue our work, protein level needs to be taken in account as it is possibly that expressed mRNA of investigated TLRs was not translated to active proteins. Moreover, different PRRs can be included in the research with selected non-transformed cell lines. SNP analysis of bacterial genome may be useful in determining the genetic differences between strains from same PFGE group that were obtained from the French outbreak and behaved differently in some assay. This could give evidence that these strains may vary virulence factor production due to this SNP differences, which will improve our understanding of bacterial virulence.

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