Cronobacter sakazakii clinical isolates overcome host barriers and evade the immune response

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Abstract

Cronobacter sakazakii is the most frequently clinically isolated species of the *Cronobacter* genus. However the virulence factors of *C. sakazakii* including their ability to overcome host barriers remains poorly studied. In this study, ten clinical isolates of *C. sakazakii* were assessed for their ability to invade and translocate through human colonic carcinoma epithelial cells (Caco-2) and human brain microvascular endothelial cells (HBMEC). Their ability to avoid phagocytosis in human macrophages U937 and human brain microglial cells was investigated. Additionally, they were tested for serum sensitivity and the presence of the *Cronobacter* plasminogen activation gene (*cpa)* gene, which is reported to confer serum resistance.

Our data showed that the clinical *C. sakazakii* strains invaded and translocated through Caco-2 and HBMEC cell lines and some strains showed significantly higher levels of invasion and translocation. Moreover, *C. sakazakii* was able to persist and even multiply in phagocytic macrophage and microglial cells. All strains, except one, were able to withstand human serum exposure, the single serum sensitive strain was also the only one which did not encode for the *cpa* gene. These results demonstrate that *C. sakazakii* clinical isolates are able to overcome host barriers and evade the host immune response indicating their capacity to cause diseases such as necrotizing enterocolitis (NEC) and meningitis. Our data showed for the first time the ability of *C. sakazakii* clinical isolates to survive and multiply within human microglial cells. Additionally, it was shown that *C. sakazakii* clinical strains have the capacity to translocate through the Caco-2 and HBMEC cell lines paracellularly.

Keywords: *Cronobacter sakazakii*, microglia, meningitis, necrotising enterocolitis

1. Introduction

The *Cronobacter* genus is a member of the *Enterobacteriaceae* family. It comprises a distinct group of Gram-negative bacilli that are catalase-positive, oxidase-negative, non-spore forming, facultatively anaerobic, and motile via peritrichous flagella [1-3]. The *Cronobacter* genus contains 7 different species including *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis* [4-6]. *C. sakazakii* isolates represent 72.1% (n=1400) of the total *Cronobacter* genus isolates in the open access *Cronobacter* PubMLST database (http://www.pubmlst.org/cronobacter/), and this species has been linked to several fatal NEC and meningitis cases around the world [7-10]. *C. sakazakii* have been isolated from prepared infant feeds associated with neonatal intensive care unit (NICU) infections. Cases of necrotising enterocolitis (NEC), bacteraemia, and meningitis have a 40-80% mortality rate, and 20% of the survivors develop serious neurological disorders [11-14]. *C. sakazakii* distinct pathovars which are clonal lineages, of particular clinical significance being clonal complex 4 (CC4) that contains sequence type 4 (ST4), as well as ST12. These are strongly associated with invasive meningitis and NEC cases, respectively [15]. One of the most studied NICU outbreaks was in 1994 when 3 infants died from infections by *C. sakazakii* ST4 strains [14].

For organisms to establish a systemic infection they must adhere to the host cell, translocate to the underlying tissues, and then disseminate throughout the body. Therefore, the intestinal epithelium has an important role in protecting the body against bacterial invasion. Once this layer loses its integrity, the invading organism can infect the tissue beneath [16]. The ability of *C. sakazakii* to invade the intestinal epithelium and brain endothelium is therefore a crucial step for its pathogenesis. It was shown previously that *C. sakazakii* has the ability to adhere to epithelial and endothelial cells *in vitro* [13, 17]. A study by Townsend et al. [18] used isolates from the French outbreak in 1994, and showed that the *C. sakazakii* strains were able to adhere and invade Caco-2 and rat brain capillary endothelial cells (rBCEC4) cell lines. Moreover, the organism was able to persist and multiply within the human macrophage U937 cell line [19]. Another study by Giri et al. [12] showed that food and environmental strains of *C. sakazakii* have the ability to invade the HeLa subline

INT407 (human embryonic intestinal cells) and human brain microvascular endothelial cells (HBMEC).

The translocation process of the organism follows the initial attachment and invasion phases. It is the step that initiates the pathogenesis at the next tissue level after passing through the epithelial layer. Townsend et al. [20] reported that the presence of lipopolysaccharide (LPS) in infant formula increased the permeability of the intestinal epithelium leading to the translocation of *C. sakazakii*. Giri et al. [12] showed that the invasive food and environmental *C. sakazakii* strains were able to translocate intracellularly through the intact monolayers of the Caco-2 and HBMEC cell lines. This suggests that the bacterium is able to overcome the physical host barriers in intestines and CNS.

A number of virulence traits have been identified in *Cronobacter*, which may facilitate the invasion and dissemination of the organism in the host. Franco et al. [21] reported that the plasmid-borne *Cronobacter* plasminogen activator (Cpa) may provide resistance to bactericidal activity of serum through cleaving complement components C3 and C4b, and the activation of plasminogen and inactivation of α2-AP. In a study of over 100 *Cronobacter* genomes, *cpa* was found in *C. sakazakii* and not *C. malonaticus* [23; http://pubmlst.org/cronobacter/], and therefore may contribute to the higher clinical incidence of this species. It has also been reported that the outer membrane protein A (OmpA) of *Cronobacter* spp. has a role in the colonisation of the gastrointestinal tract (GIT) [21, 23]. Also, it was demonstrated that the outer membrane proteins OmpA and OmpX were required for the basolateral invasion of enterocyte-like human epithelial cells by *C. sakazakii* [23]. Singamsetty et al. [24] demonstrated that the entry of *Cronobacter* spp. into HBMEC requires *ompA* expression and depends on microtubule condensation in these cells. This might help in the invasion of human intestinal cells and invasion of the brain endothelial cells to cause meningitis [25]. Moreover, it was recently shown that *C. sakazakii* ST4 strain 767 was able to produce outer membrane vesicles (OMVs) that have the capacity to increase the host's cell prolifration and stimulate a pro-inflammatory innate immune response [26].

This study used clinical isolates of *C. sakazakii* which had been previously genotyped by multilocus sequence typing (MLST), and many of which had been whole genome sequenced [23; http://pubMLST.org/cronobacter/]. The research aim was to study the virulence potential and pathogenicity of well characterised *C.*

sakazakii clinical isolates and their ability to overcome host physical barriers and evade host immune response.

2. Results

2.1. Invasion efficiencies of C. sakazakii clinical isolates to Caco-2 and HBMEC cell lines

The invasion assay, using gentamicin protection to kill the extracellular bacteria, was used to assess the ability of 10 *C. sakazakii* clinical isolates to invade the Caco-2 and HBMEC cell lines. With regard to the Caco-2 cell line, different invasion levels were noted among these isolates, and strain 695 was the most significant ($P<0.05$). The level of invasion by 695 was as high as *S*. Enteritidis, which was used as positive control strain for the assay. Strains 20, 767, 1221, and 696 were moderate in invasion, whereas strains 1240, 1242, 1249, 658 and 680 were low (Fig.1). Regarding the HBMEC cell line invasion, strain 767 was the most significant ($P<0.01$) being as high as *Cit. koseri*, the positive control. The other strains were moderate except for strains 658 and 680, which were the lowest (Fig.1).

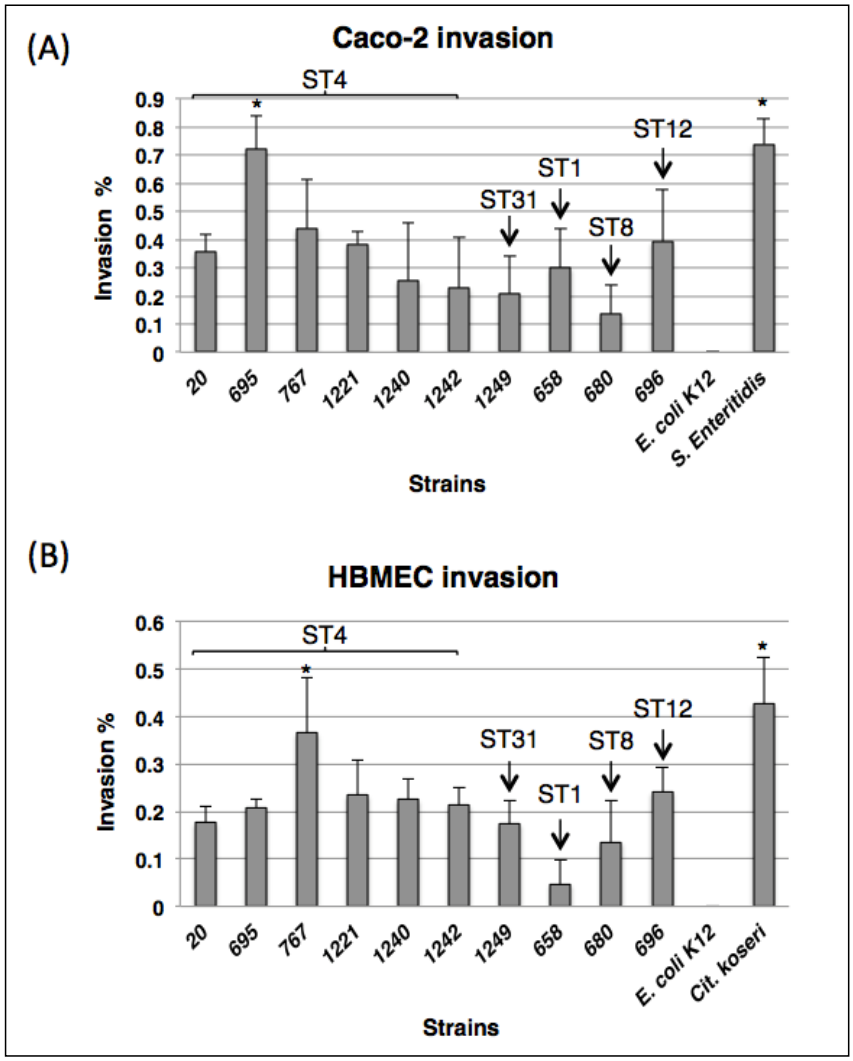

Fig.1. *C. sakazakii* invasion assay using Caco-2 (A) and HBMEC (B) cell lines over 3 hours of incubation showing the differences in invasion levels among strains. The displayed data are the mean±standard deviation of invasion efficiency % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences (*P<0.05; Kruskal-Wallis).

175 *2.2. Translocation of C. sakazakii clinical through Caco-2 and HBMEC polarised*
176 *monolayers*
177
178 The aforementioned results showed that *C. sakazakii* isolates were able to invade
179 Caco-2 and HBMEC monolayers. Therefore, these isolates were tested for their
180 ability to translocate through the polarised monolayers of the Caco-2 and HBMEC
181 cell lines. *C. sakazakii* strain 695 was the highest in translocating through the Caco-2
182 cell line over 5 hours of infection (P<0.01). The other strains including 767, 1221,
183 1240, 1242, 1249, 658, and 696 were moderate, while strains 20 and 680 were the
184 lowest (Fig.2). With regard to the HBMEC cell line, strains 20, 695, 1221, 1240, and
185 696 were high in translocation (P<0.01), and strain 767 was the most significant over
186 5 hours of incubation (P<0.001). *C. sakazakii* strains 1242 and 1249 were moderate,
187 whereas 658 and 680 were the lowest (Fig.2). It was noted that the transepithelial
188 electrical resistance (TEER) declined over the period of the experiment. The higher
189 drops of TEER were accompanied with high translocation levels (Fig.3), suggesting
190 that *C. sakazakii* clinical isolates might translocate through the Caco-2 and HBMEC
191 cell lines paracellularly.
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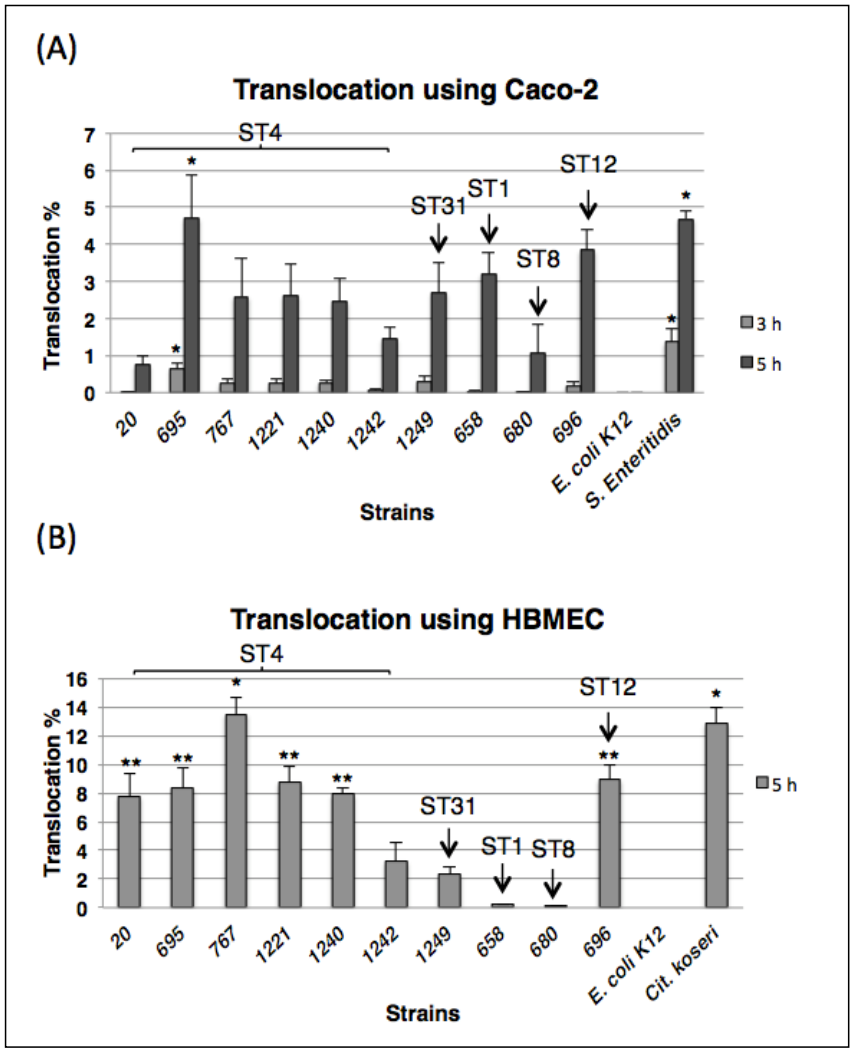


Fig.2. *C. sakazakii* translocation assay using Caco-2 (A) and HBMEC (B) cell lines over 5 hours of incubation showing the differences in translocation ability among strains. The displayed data are the mean±standard deviation of translocation efficiency % of the initial inoculum (10^6 cfu/ml) of two independent experiments. The asterisks above the bars indicate statistically significant differences (*P<0.001, ** P<0.01; Kruskal-Wallis).

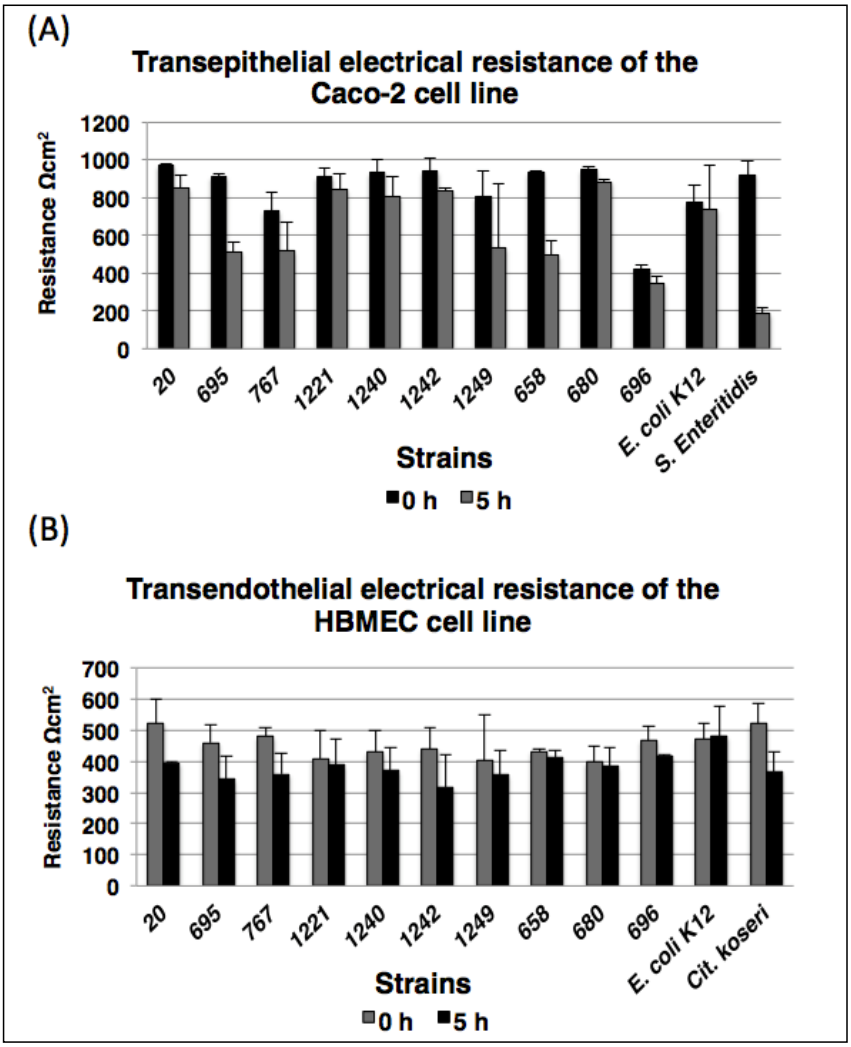


Fig.3. Transepithelial electrical resistance (TEER) of the Caco-2 cell line (A) and the transendothelial electrical resistance (TEER) of the HBMEC cell line (B) over 5 hours of incubation, showing changes in resistance over time.

2.3. C. sakazakii cytotoxic effect on the Caco-2 and HBMEC cell lines

C. sakazakii clinical strains in this study (*n*=10) were tested for their cytotoxic impact using the MTT assay. Apart from strain 680, all the other strains were able to induce cell death of the Caco-2 cell line, as the assay displayed declined absorbance levels after 3 hours of incubation indicating low MTT reduction. Although strains 1221, 1240, and 1249 did not follow the same pattern, they however showed lower absorbance when compared to strain 680. This suggests that these strains, to some extent, are able to induce cell death more than strain 680 (Fig.4). With regard to the HBMEC cell line, it did not show susceptibility to cytotoxicity over the first hour of the assay in contrast to Caco-2 cells; nevertheless after prolonged incubation for 3 hours the cytotoxic effect appeared to be increased (Fig.4).

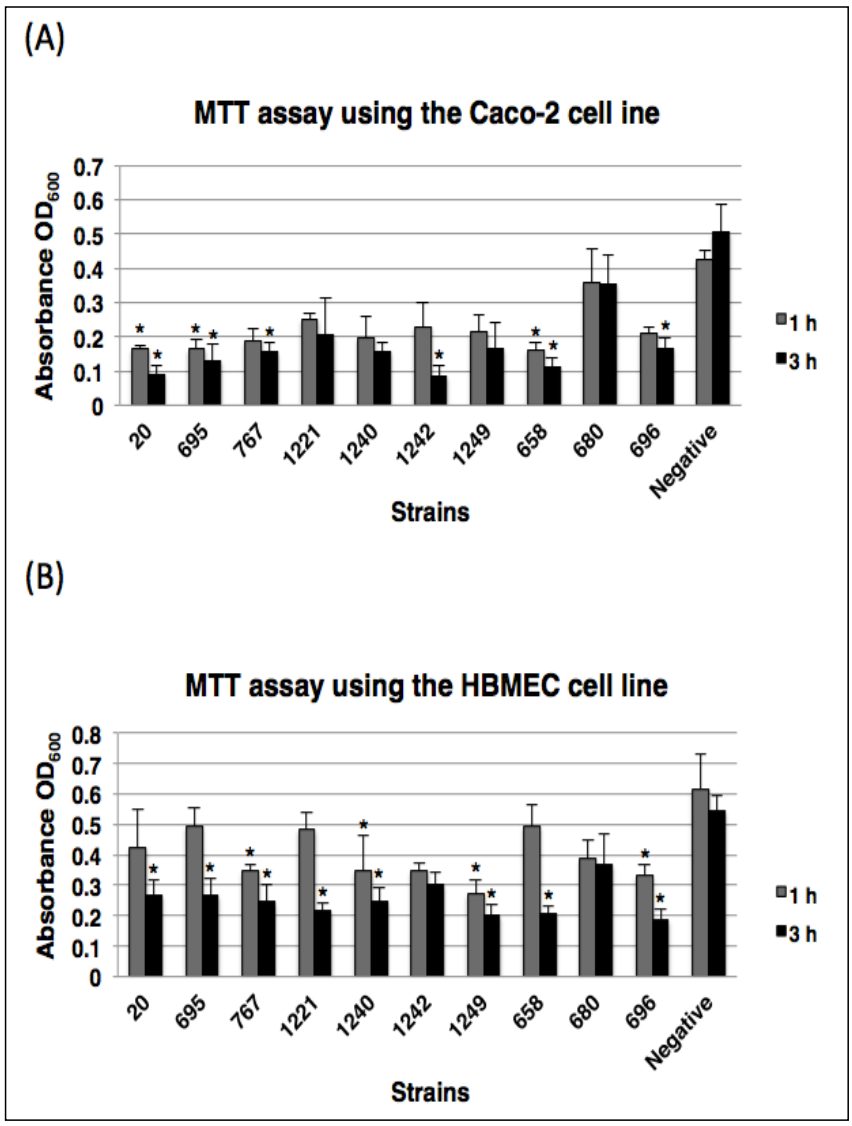


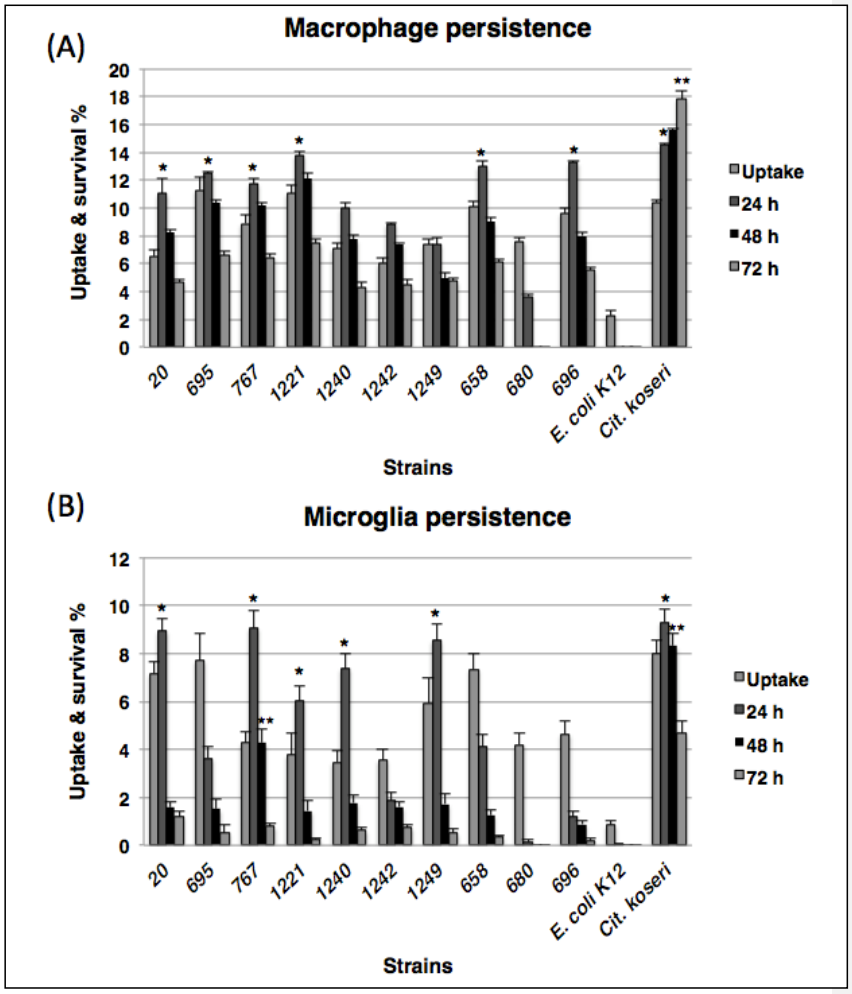
Fig.4. Cytotoxicity of *C. sakazakii* strains on Caco-2 (A) and HBMEC (B) up to 3 hours of incubation. MTT reduction was used to measure the cytotoxicity levels of *C. sakazakii* strains where only the viable HBMEC cells are able to reduce MTT to its insoluble purple form formazan, the higher absorbance (OD_{600}) the higher in MTT reduction (low toxicity) and vice versa. The negative control used was uninfected cells treated using the same protocol with no bacteria added. The data presented in mean±standard error of mean of three independent experiments. The asterisks above the bars indicate statistically significant differences between the strains in this experiment (*P<0.05; ANOVA).

2.4. C. sakazakii survival within human macrophages U937

The survival within macrophages is an important indicator of the pathogenicity of the organism. It enables the persistent bacterium to evade the immune response inside the host. *C. sakazakii* clinical isolates were tested for their ability to survive within human macrophages using U937 cell line. All strains were taken up by these cells and showed persistence for up to 72 hours (Fig.5). However, strain 680 declined significantly after 72 hours. The other strains demonstrated different levels of multiplication at 24 hours and strains 20, 695, 767, 1221, 658, and 696 were the most significant (P<0.05).

2.5. C. sakazakii survival within human microglial cells

Microglial cells are brain resident macrophages, which respond rapidly to the presence of the pathogens and brain damage. Furthermore, they perform phagocytosis, antigen presentation, and are responsible for cytokine secretion. Microglial cells are able to migrate to the injured brain tissues to remove the damaged ones [27]. *C. sakazakii* is linked to fatal meningitis cases, and therefore it is important to consider its ability to resist phagocytosis and withstand killing inside the brain. This experiment was conducted to assess the ability of *C. sakazakii* to survive within microglial cells and multiply intracellularly. *C. sakazakii* clinical strains were able to survive up to 72 hours post infection (Fig.5). However, strains 695, 1242, 658, and 696 showed lower survival levels. Additionally, strain 680 was taken up and killed rapidly following the uptake. Strains 20, 767, 1221, 1240, and 1249 multiplied significantly at 24 hours (P<0.001).


(A)
Macrophage persistence
Uptake & survival %
20
18
16
14
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695
767
1221
1240
1242
1249
658
680
696
E. coli K12
Cit. koseri
Strains
Uptake
24 h
48 h
72 h
(B)
Microglia persistence
Uptake & survival %
12
10
8
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4
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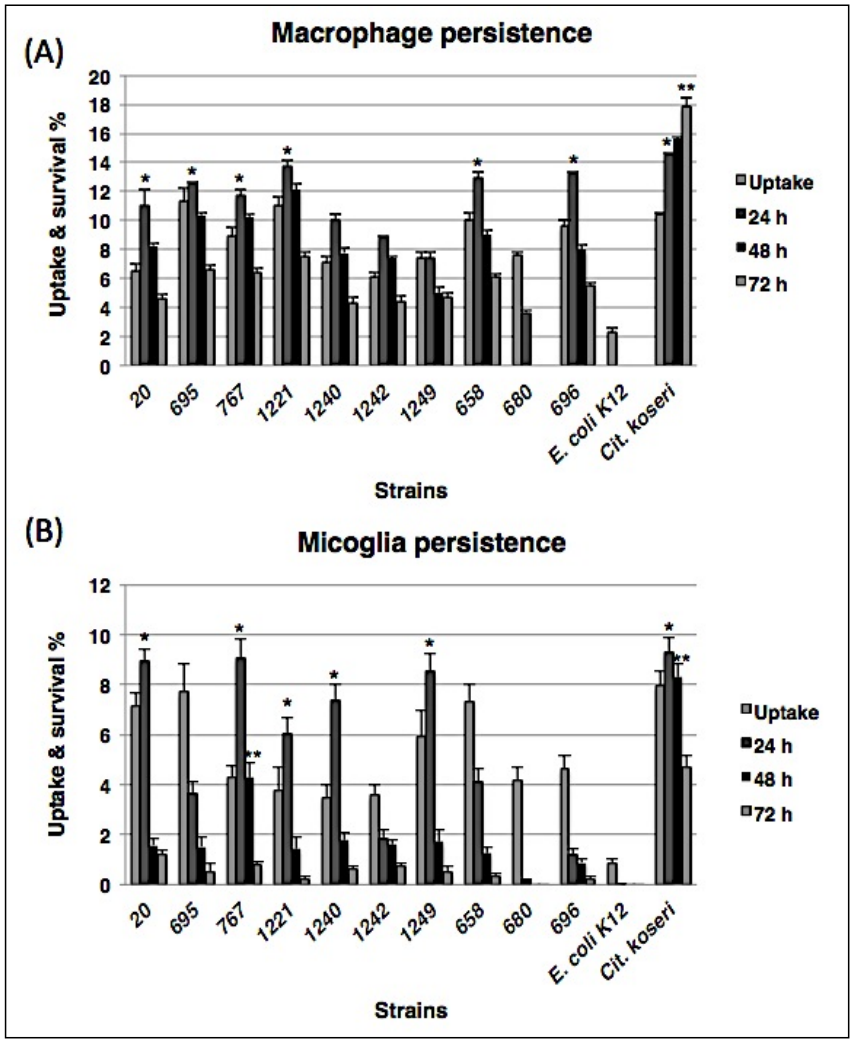

Fig.5. *C. sakazakii* uptake and persistence assay using human macrophage (A) and human microglia (B) cell lines over 72 hours of incubation showing the differences in survival among strains. The displayed data are the mean±SEM for uptake and persistence efficiency % of the initial inoculum (10^5 cfu/ml) of three independent experiments. The asterisks above the bars indicate statistically significant differences (*P<0.05, **P<0.001; ANOVA).

2.6. C. sakazakii resistance to human serum

All *C. sakazakii* strains in this experiment (*n*=10) showed up to 60% decrease in their viable count at the first hour incubation with human serum (Fig.6). After the second hour of incubation, it was noted that 9/10 strains showed an increase in their viable numbers, whereas strain 680 showed a 30% reduction in its viability. The majority of the strains were able to survive in human serum and increased by up to 4-fold in their numbers after 3 hours of incubation. The exception was strain 680, which was serum sensitive and its growth declined dramatically to 60% ($P<0.05$). Strains 20, 1242, 1249, and 696 were the highest in serum tolerance showing considerable elevated growth rates (>200%) during the period of the assay.

Franco et al. [21] reported that the outer membrane protease Cpa of *Cronobacter* is responsible for serum resistance. All *C. sakazakii* clinical strains in this research were tested for the presence of the *cpa* gene using PCR probing and BLAST search. Most (9/10) of the strains were confirmed positive for this gene. These strains demonstrated resistance to human serum except for strain 680, which lacked the presence of *cpa* gene in PCR and BLAST search and was serum sensitive. It was shown that strain 680 lack the presence of pESA3 plasmid, but on the other hand a pESA3-like plasmid was present in this strain, which lacked some plasmid-borne virulence genes [22].

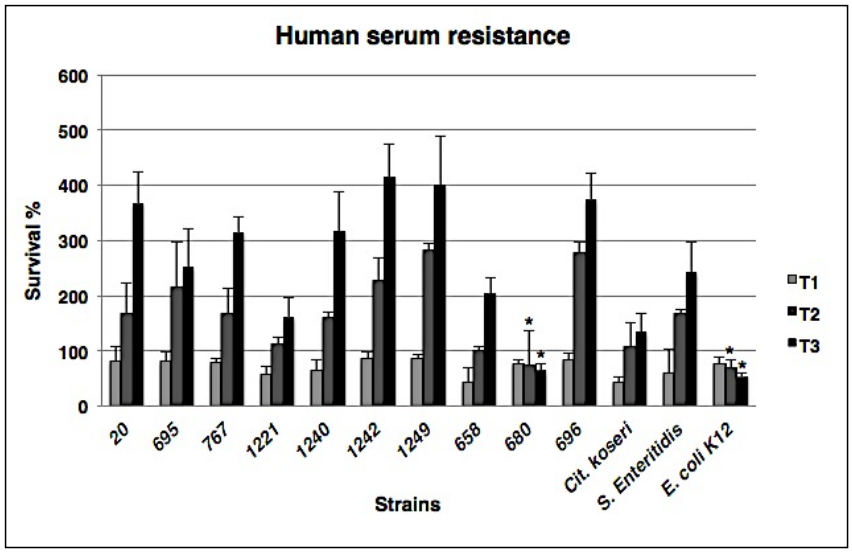

Fig.6. Sensitivity of *C. sakazakii* to human serum over 3 hours of incubation showing the difference in growth among strains overtime. Most of the strains showed increases in their viable counts, and strains 6 (ST4), 680 (ST4), and *E. coli* K12 showed significantly declined values. The displayed data are the mean±standard deviation of survival % (10^6 cfu/ml initial inoculum) of two independent experiments. The asterisks above the bars indicate statistically significant differences (*P<0.05; Kruskal-Wallis).

3. Discussion

This study used a range of clinical isolates of *C. sakazakii*. These strains were diverse with respect to their temporal and geographical sources, and all but one strain were from patients with clinical presentations. The exception was *C. sakazakii* 658 (ATCC BAA-894) which had been isolated from the formula, that was not intended for infants, used in the fatal NICU *C. sakazakii* outbreak at the University of Tennessee [7]. The corresponding CSF isolate has not been deposited in any international culture collection and therefore the PFGE indistinguishable strain (ATCC code BAA-894) was used instead. This was also the first genome-sequenced strain of *Cronobacter,* including the pESA3 plasmid which encodes for the *cpa* gene [2].

Tissue culture assays were applied to examine the bacterial-host interaction and to assess the ability of *C. sakazakii* strains to overcome human intestinal and brain barriers represented by the Caco-2 and HBMEC cell lines. All the strains were able to invade the Caco-2 cell line (Fig.1). However, there was strain to strain variation in the level of invasion, with strain 695 (ST4) being the most invasive and was similar to that of the positive control *S*. Enteritidis strain ($P<0.05$). With reference to HBMEC cell line invasion, most of the strains showed moderate invasion levels and strain 767 (ST4) displayed significant high invasion level ($P<0.01$). Whereas, strains 658 (ST1) and 680 (ST8) showed the lowest invasion (Fig.1).

Overall, the invasion results indicated that the majority (9/10) of the strains were able to invade the cell lines and therefore potentially translocate towards the underlying tissues and blood stream. Consequently, this could lead to the dissemination of the organism around the body. To further investigate the translocation process further, assays using the same human cell lines were applied. All strains were able to translocate through the Caco-2 cell line especially strain 695 that displayed significant high translocation ability ($P<0.01$), while strain 696 (ST12) was moderate (Fig.2). These strains were able to cause high cytotoxicity levels to the Caco-2 cell line, which could lead to increase the cell line permeability causing more bacterial translocation (Fig.4). Strain 695 (ST4) accounted for a fatal neonatal NEC infection while strain 696 (ST12) was previously linked to neonatal NECII infection [14]. The invasion and translocation results correlate their virulence and their ability to cause that disease.

There was a significant decrease in TEER when using the invasive and translocating strains indicating a disruption in the tight junctions (Fig.3). This might explain the mechanism of translocation for the strains where they can migrate in between the cells by altering the tight junctions. This might trigger the onset of NEC, which could lead to the spread of the bacteria in the blood stream. This clinical presentation of the organism indicates the potential to reach the brain microvascular endothelium and invades its cells. Therefore the translocation assay using the HBMEC cell line was initiated to investigate whether the organism has the ability to translocate through these cells or not. ST4 strains 20, 695, 1221, and 1240 in addition to 696 (ST12) were high in translocation ($P<0.01$), and strain 767 (ST4) was the most significant in translocation over 5 hours of incubation ($P<0.001$; Fig.2). *C. sakazakii* strains 1242 (ST4) and 1249 (ST31) were moderate in translocation, whereas 658 (ST1) and 680 (ST8) were the lowest. Strains 695 (ST4), 767 (ST4), and 696 (ST12), which showed

high translocation rates were the highly cytotoxic strains to the HBMEC cell line (Fig.4). Strain 767 (ST4) that exhibited the most significant translocation through HBMEC cell line was associated with a fatal meningitis case [14] and also produces cytopathogenic OMVs [26]. Overall, the HBMEC TEER was stable during the first 4 hours of incubation, indicating the stability of the tight junctions and the integrity of the cell line. However, after 5 hours of infection it displayed declined levels with the translocated strains (Fig.3). The decrease in TEER might be attributed to the deterioration of endothelial integrity that could be accredited to the cytotoxic killing to the HBMEC cell line that was caused by those strains.

The translocated strains might need a longer infection period to accomplish the translocation process, as the translocation was noted 5 hours post infection, while in Caco-2 it was after 1 hour with some strains. Moreover, they could use different mechanisms of invasion to overcome this barrier that is a part of the BBB. One of the possible mechanisms that might assist the strains to translocate is the bacterial cytotoxicity that leads to initiate cell death in the cell line via apoptosis or necrosis. Moreover, the cytokines released by the cells might play a role in the same process by making the cell line permeable. High levels of nitric oxide (NO) are a potential factor that could contribute in the permeability of the cell line. Also, OMVs might contribute in this process by triggering the host proinflammatory response leading to the secretion of some inflammatory mediators, including cytokines and NO, that in turn could cause cell line permeability [26]. The translocation of these strains indicated their potential to pass through towards the brain tissues triggering the host response, which could result in brain inflammation and tissue damage.

In a previously published study by Giri et al. [12], non-clinical *C. sakazakii* isolates were examined and showed their ability to invade and translocate through the Caco-2 and HBMEC cell lines intracellularly. In this research, the same cell lines were used to compare the results of the clinical strains in our study and the non-clinical strains from the previous research by Giri et al. [12] with regard to the capacity and mechanism of translocation. ~~However, the results~~We found that ~~of~~ the ~~capacity of translocation~~translocation ability of *C. sakazakii* clinical isolates ~~that were obtained by our research were~~ was more than 10 times higher than the previous study using the same cell lines. Additionally, our results ~~showed~~ suggested a different mechanism of translocation, which is~~their capacity of~~ paracellular translocation through those cell lines. All of the strains were from clinical sources and were linked to severe and fatal neonatal cases.

The survival studies were conducted to investigate the ability of the test strains to survive and multiply within macrophages and microglia which are both types of immune cells. *C. sakazakii* strains used in the survival experiments showed the ability to persist within human macrophages cell line U937 for up to 72 hours of incubation. Moreover, ST4 strains 20, 767, and 1221 in addition to 658 (ST1) were able to survive and multiply significantly ($P<0.001$). The survival results were comparable to the results obtained previously by Townsend et al. [18]. Strains 767 (ST4) and 696 (ST12) showed similar persistence and multiplication levels as the ones published in that research. However, Townsend et al. [18] reported that strain 695 (ST4) was able to survive and could not multiply within macrophages. These results are in contrast of the ones obtained here as strain 695 (ST4) showed the ability to survive and multiply in U937 cells. Our results correlate the virulence potential of the strain, and the invasion and translocation profiles, as it was an invasive strain to both of the Caco-2 and HBMEC cell lines. Moreover, it is a clinical strain that was linked to a fatal NEC infection [14]. This suggests that this strain was able to establish a successful infection and has the virulence traits to avoid phagocytic killing.

The survival and multiplication within macrophages could help the organism to use macrophages as a vehicle to invade the other body organs. This mechanism is called the “Trojan horse” and where the organism translocates through tissues inside macrophages. This mechanism allows the bacterium to hide inside the phagocytic cells, escape from the immune response, and reach the other body organs such as the brain [28]. Some cytokines secreted by the infected tissues, such as IL-8, attract phagocytic cells and make these tissues permeable and leaky allowing the immune cells to migrate to the site of infection, and help in increasing the number of the invading organism [29-31]. The damage could be indicated by the host response induced by the bacterium. Moreover, the persistent strains were confirmed to be serum resistant, and these two characteristics enhanced their ability to avoid the host immune response and cause bacteraemia, which could be advantageous for the organism to migrate through the BBB endothelium.

Microglia are brain resident innate immune cells that are responsible for phagocytosis as well as the ability to produce inflammatory mediators such as NO and TNF-α [27, 32, 33]. Although their ability to eliminate *C. sakazakii* CNS infection *in vitro* has not yet been examined, we are the first to report the ability of *C. sakazakii*

to survive within human microglial cells. It was shown in this research that the majority of *C. sakazakii* strains were able to persist in human microglia as represented by the HMGC cell line for 72 hours. Moreover, ST4 strains 20, 767, 1221, and 1240 in addition to 1249 multiplied significantly in this cell line (P<0.001). Although these strains showed the ability to multiply within microglia, they nevertheless showed declined levels of persistence afterwards. The ability of the bacteria to reproduce intracellularly within these phagocytic cells demonstrates their virulence potential to withstand the bactericidal activity of microglia and evade the host immune response.

Invasive microorganisms have protective mechanisms against serum-mediated killing. Bacterial structures including outer membrane proteins and proteases were identified for their roles to avoid this bactericidal action [34-36]. Franco et al. [21] showed that the Cpa is a plasminogen activator that plays an essential role in *Cronobacter* serum resistance. Nine of the ten *C. sakazakii* strains in this research were regarded as serum resistant being able to replicate in human serum and appeared to be completely refractory to serum killing. Whereas *C. sakazakii* strain 680 was serum sensitive and showed significant reduction in viability (P<0.05; Fig 6).

Franco et al. [21] further showed that a *cpa* mutant of *C. sakazakii* BAA-894 (synonym for 658 in this study) was serum sensitive compared with the wild type. In our study 9/10 *C. sakazakii* strains were serum resistant and also encoded for the *cpa* gene. The exception being strain 680 which lacked this gene and was serum sensitive. While strain 680 was the only ST8 strain studied here, a BLAST search of the PubMLST database revealed the *cpa* gene is absent from all (n=8) genome sequenced ST8 strains. This absence could explain the observation by Forsythe et al. [15] in a review of >1000 *Cronobacter* strains, that *C. sakazakii* ST8 is not associated with severe *Cronobacter* infections [8]. By inference therefore, *cpa* could be an important factor in *C. sakazakii* resistance to serum killing in the host and enabling dissemination around the neonate's body.

In conclusion, *C. sakazakii* clinical isolates showed the ability to invade and translocate through Caco-2 and HBMEC cell lines. Moreover, they demonstrated the ability to persist and multiply within macrophages and microglial cells. Additionally, the isolates were resistant to human serum bactericidal effect. However, it was noted that strain 680 did not follow the same pattern of virulence and pathogenicity as it was low in invasion, translocation, and phagocytosis survival in addition to being

sensitive for human serum killing. This might be attributed to lack of the pESA3 plasmid [22], and hence other plasmid-borne virulence genes such as *cpa,* and other genes involved in invasion, translocation, and phagocytosis survival . Although this strain was a CSF isolate it demonstrated a weakness in pathogenicity to HBMEC and microglial cells *in vitro*. It was reported that *C. sakazakii* ST12 has been associated with cases of necrotizing enterocolitis [15]. It was shown in this study that strain 696 (ST12) was able to invade and translocate through the Caco-2 cell line in addition to its ability to induce cytotoxicity to the same cell line. This might indicate its ability to induce necrotizing enterocolitis *in vivo*. Furthermore, it was observed that the most invasive and translocated strains in this research were in the *C. sakazakii* ST4 clonal complex, which is the lineage that linked to the most neonatal meningitis cases worldwide [37]; Table 1. ST4 strains such as 695, 767, 1221, 1240, and 1242 within clonal complex 4 were invasive and highly translocated isolates and were responsible for fatal NEC and meningitis infections. This indicates their ability to translocate through the gut mucosa and BBB *in vivo* and cause NEC and meningitis. It is important to further study the strains among this clonal complex and discover their pathogenicity traits and their role in triggering the host response and its outcome.

4. Materials and methods

4.1. Bacterial strains

Ten *C. sakazakii* strains were used in this research (Table.1). These isolates were from Nottingham Trent University culture collection. Additional metadata for all strains can be obtained from the open access *Cronobacter* PubMLST database: www.pubmlst.org/cronobacter/. The strains were chosen according to their source and clinical outcomes, and well-characterised strains from the 1994 French outbreak and University of Tennessee [7, 14]. For routine culturing the strains were grown on TSA (Oxoid, UK) under aerobic conditions at 37°C for overnight.

Table.1

Cronobacter sakazakii strains used in this study

Strain	Sequence type (clonal complex)	Source	Clinical presentation	Country
20	4 (CC4)	Clinical	Unknown	Czech Republic
695	4 (CC4)	Clinical - Trachea	Fatal NEC II	France
767	4 (CC4)	Clinical - Trachea	Fatal meningitis	France
1221	4 (CC4)	Clinical - CSF	Meningitis	USA
1240	4 (CC4)	Clinical - CSF	Fatal meningitis	USA
1242	4 (CC4)	Clinical - Brain	Fatal meningitis	USA
1249	31 (CC31)	Clinical	Fatal infant isolate	UK
658[a]	1 (CC1)	~~NIF~~NIF	Meningitis	USA
680	8 (CC8)	Clinical - CSF	Unknown	USA
696	12	Clinical - Faeces	NEC II	France

NEC: Necrotising enterocolitis. CSF: Cerebrospinal fluid. CC: clonal complex. NIF: Non-infant formula.
a Also known as *C. sakazakii* BAA-894.

4.2. Cell culture

Human colonic carcinoma epithelial cells (Caco-2) passages 17 to 45 acquired from the European Collection of Cell Cultures (ECACC #86010202) and human brain microvascular endothelial cells passages 2 to 25 (HBMEC; ref. #HMG030 Inooprot, Spain). Macrophage cell line (U937) passage 12 was obtained from American Type Culture Collection (ATCC; #CRL-1593.2), and human microglial cell line passage 3 was obtained from Innoprot Technologies (Ref.# P10354). All experiments were applied at consistent conditions of time, temperature, cell line passage, mammalian cells concentration, and bacterial suspension. *Salmonella* Enteritidis strain NTU 358 was used as positive control for Caco-2 cell line, while *Citrobacter koseri* strain NTU 48 was the positive control for HBMEC, macrophages, and microglial cell lines. *Escherichia coli* K12 strain NTU 1230 was the negative control for all cell lines.

4.3. Bacterial invasion of mammalian cells

This experiment was as described previously by Townsend et al. [18] with slight modifications. Caco-2 cells were grown in Minimum Essential Medium (MEM) supplied with 10% (v/v) foetal calf serum (FCS), 1% (v/v) non-essential amino acid (NEAA), and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK). HBMEC were

grown in Dulbecco's modified eagle medium (DMEM) with 10% (v/v) FCS and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK). Mammalian cells then were seeded at $4x10^4$ cell/well in growth medium for 48 hours in 5% CO_2 at 37°C to achieve a confluent monolayer. *C. sakazakii* strains were grown in LB broth for overnight at 37°C. The suspension then was added to the wells at MOI 100, and incubated in 5% CO_2 at 37°C for 2 hours. The wells were then washed using PBS (Sigma Aldrich, UK). Then, 0.5 ml of infection medium supplied with 125 µg/ml (v/v) of gentamicin (Sigma Aldrich, UK) was added and incubated in 5% CO_2 at 37°C for 1 additional hour. The wells were then washed with PBS (Sigma Aldrich, UK) before lysing by 1% (v/v) Triton X-100 (Fisher Scientific, UK), and plated on TSA at 37°C for overnight incubation after serial dilution to obtain viable count. Data are presented as the percentage efficiency of invasion.

4.4. Translocation assay using Caco-2 cell line

Translocation assay was performed as previously described [12, 38]. However, 0.8 ml of growth medium was added to the basolateral chamber of Millicell-24 cell culture plate (Millipore, UK). Caco-2 cells, at a concentration of $4x10^4$ cell/well in 0.4 ml/well of Minimum Essential Medium (MEM) supplied with 10% (v/v) foetal calf serum (FCS), 1% (v/v) non-essential amino acid (NEAA), and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK), were seeded onto a 3 µm pore polycarbonate transwell membrane in the apical chamber of the tissue culture plate and incubated in 5% CO_2 at 37°C. The medium in the apical and the basolateral chambers was changed every 3 days. Millicell ERS-2 Volt-Ohm Meter (Millipore, UK) was used to measure the TEER. The Caco-2 cell line required up to 21 days to form intact polarised monolayers with TEER 300-850 Ωcm^{-2} [12, 38, 39]. On the day of the assay, the medium in the basolateral chamber was replaced with infection medium. The medium in the apical chamber was removed, and the membrane was washed using 0.4 ml of PBS (Sigma Aldrich, UK). Bacterial suspensions was prepared was added at MOI 100 to the apical chamber. At each time point of incubation, the basolateral chamber was sampled for viable count after serial dilution and inoculation on TSA. The TEER was measured at each time point. Data are presented as the percentage efficiency of translocation.

4.5. Translocation assay using HBMEC cell line

This assay was carried out using the protocol described previously [12, 40, 41]. The basolateral chambers of the 24-well plate were filled with 0.510 ml/well of Dulbecco's modified eagle medium (DMEM) with 10% (v/v) FCS and 1% (v/v) penicillin-streptomycin (Sigma Aldrich, UK). Cells, with a concentration of $4x10^4$ cell/well in 0.375 ml/well of previous medium, were seeded onto the apical part of collagen-coated polytetrafluoroethylene (PTFE) membrane with a pore size of 0.4 µm (Transwell-COL; Corning, USA), and incubated in 5% CO_2 at 37°C. The medium in the apical chamber was changed every 3 days. The transendothelial electrical resistance (TEER) was measured using Millicell ERS-2 Volt-Ohm Meter (Millipore, UK). According to electrical resistance measurements, HBMEC cell line required 5 to 8 days to form intact polarised monolayers with TEER 300-600 Ωcm^{-2} [12, 42]. Prior to infecting the cell line, the filter was washed by 0.375 ml/well PBS (Sigma Aldrich, UK), and the medium in the basolateral part was replaced by infection medium. The cell line was infected by 0.375 ml per well bacterial suspension with MOI of 100. The basolateral chamber was sampled and serially diluted and then plated for viable count on TSA at 37°C before replacing with a fresh infection medium. Moreover, the TEER of the monolayers was measured at each time point. Data are presented as the percentage efficiency of translocation.

4.6. C. sakazakii cytotoxic effect on the Caco-2 and HBMEC cell lines

The ability of *C. sakazakii* to induce cytotoxicity was assessed using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. This assay is based on the reduction of MTT (Sigma Aldrich, UK) by viable cells to its insoluble form formazan, which has a purple colour. This experiment was applied as described previously [43, 44]. Briefly, bacterial suspensions at a concentration of $4x10^6$ cfu/well (MOI 100) were added to confluent monolayers of the Caco-2 and HBMEC cell lines. The plates were then incubated in 5% CO_2 at 37°C for 1 and 3 hours. A volume of 50 µl of MTT was added per 500 µl of culture medium. Next, the medium containing MTT was removed and formazan was solubilised in dimethyl sulfoxide (DMSO; Fisher Scientific, UK). The absorbance was measured at 600_{nm} after 3 hours. The negative control for the assay consisted of uninfected cells.

4.7. C. sakazakii persistence in human macrophages

As previously given by Townsend et al. [19] with slight modifications, macrophages were grown in RPI medium containing 10% (v/v) FCS, 1% (v/v) NEAA, and 1% (v/v)

705 penicillin-streptomycin (Sigma Aldrich, UK), and then treated with growth medium
706 contains 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate (Sigma
707 Aldrich, UK) for maturation. Before seeding the 24-well plates, phorbol 12-myristate
708 13-acetate (PMA; Sigma Aldrich, UK) at a concentration of 0.1 µg/ml (v/v) was added
709 for promoting cell adhesion. The cells were then plated into 24-well plates at a
710 concentration of $4x10^4$ cell/well and incubated in 5% CO_2 at 37°C for 72 hours to get
711 confluent monolayer. Macrophages were infected by overnight-cultured bacterial
712 suspensions with MOI 10. The plates then were incubated 1 hour in 5% CO_2 at 37°C.
713 After the previous incubation period, the medium was aspirated and replaced with
714 infection medium containing 125 µg/ml (v/v) of gentamicin and incubated in 5% CO_2
715 at 37°C for 1 hour. Four plates were then washed by PBS and supplied with infection
716 medium contains 50 µg/ml (v/v) of gentamicin for further incubation. After each time
717 point of incubation, the plates were washed by PBS before lysing by 1% (v/v) Triton
718 X-100, and then serially diluted before plating on TSA to obtain the intracellular
719 bacteria at different time points. Data are displayed in percentage of uptake and
720 persistence.
721
722 *4.8. C. sakazakii persistence in human microglial cells*
723
724 As previously described by Liu et al. [45] with slight modifications, microglial cells
725 were grown in basal medium containing 10% (v/v) FCS, 10% (v/v) microglial growth
726 supplement, and 1% (v/v) penicillin-streptomycin (Innoprot, Spain) for three days in
727 75 cm^3 tissue culture flask. The cells then were seeded into four 24-well plates at a
728 concentration of $4x10^4$ cell/well and incubated in 5% CO_2 at 37°C for 48 hours to
729 achieve confluency. Next, the cells were infected by overnight-cultured bacteria with
730 MOI 10. Afterwards, the plates were incubated for 1 hour in 5% CO_2 at 37°C. The
731 medium then was aspirated and replaced by infection medium contains 125 µg/ml
732 (v/v) of gentamicin and incubated in 5% CO_2 at 37°C for 1 hour after washing by
733 PBS. Three plates were then washed 3 times by PBS and supplied with infection
734 medium contains 50 µg/ml (v/v) of gentamicin for further incubation. At the end of
735 each time point of incubation, the cells were washed by PBS before lysing with 1%
736 (v/v) Triton X-100, and plated on TSA after being serially diluted to obtain the
737 intracellular bacteria at different time points. Data are displayed in percentage of
738 uptake and persistence.
739
740 *4.9. C. sakazakii sensitivity to human serum*
741

The sensitivity of *C. sakazakii* strains to active human serum was conducted as described previously by Hughes et al. [46] with slight modification. Bacterial cultures were grown overnight in LB at 37°C with shaking at 200 rpm then centrifuged for 10 minutes at 1300 rpm (Mikro 200-Hettik). The pellet then was re-suspended to 10^6 cfu/ml in 5 ml of phosphate buffered saline (PBS; Sigma Aldrich, UK). A volume of 0.5 ml of the suspension was added into 1.5 ml of undiluted active human serum (Sigma Aldrich, UK). The samples were loaded into a 24-well plate and incubated at 37°C. Viable counts were obtained at 4 different time points. Data are displayed in percentage of percent survival of inoculum.

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4.10. PCR probing for cpa gene and BLAST genome search

Plasmid DNA extraction was carried out according to the manufacturer's instructions using QIAprep Spin Miniprep Kit (Qiagen, UK). PCR primers were designed to target *cpa* loci on the large *C. sakazakii* plasmid pESA3 plasmid. GoTaq® DNA Polymerase kit (Promega, UK) was used for all PCR reactions' preparation. Primers design, and the PCRs were all conducted according to Franco et al. [25]. All reactions started with 3 minutes at 94°C and denaturation step at 94°C for 30 seconds, followed by 25 cycles of 30 s at 56 °C and 30 s at 72 °C. The final extension period was 10 min at 72 °C. Primer pair cpafw, 5'-GACAACCCTGAGTTCTGGTAAC, and cparv, 5'-ATGCGTATTTCTGCTGGTAA, targets a 306 bp region. Moreover, BLAST genome search was applied to strains using the sequence of *cpa* gene to confirm its presence. The BLAST search was at http://www.pubmlst.org/cronobacter.

4.11. Statistical analysis

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

statistical analysis software was used to perform the analysis (IBM SPSS version 22.0, Chicago, IL, USA).

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