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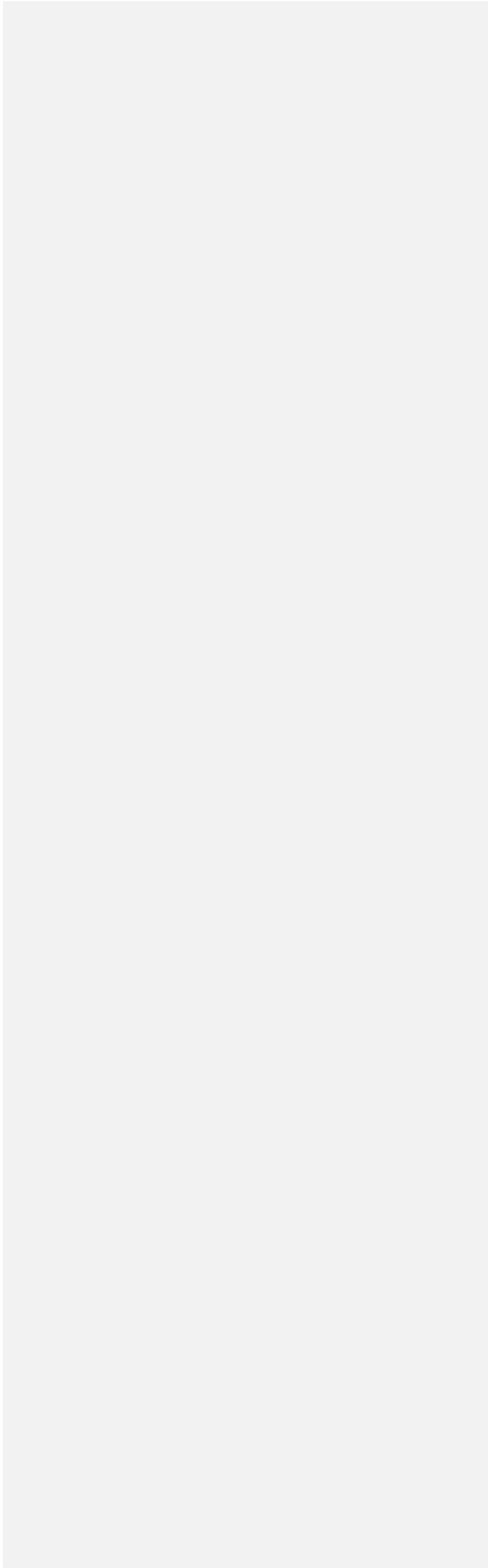
***Cronobacter sakazakii* clinical isolates overcome host barriers and evade the immune response**

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36

37 **Abstract**

38

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

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

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

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73 1. Introduction

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75 The *Cronobacter* genus is a member of the *Enterobacteriaceae* family. It comprises a
76 distinct group of Gram-negative bacilli that are catalase-positive, oxidase-negative,
77 non-spore forming, facultatively anaerobic, and motile via peritrichous flagella [1-3].

78 The *Cronobacter* genus contains 7 different species including *C. condimenti*, *C.*
79 *dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C.*
80 *universalis* [4-6]. *C. sakazakii* isolates represent 72.1% (n=1400) of the total

81 *Cronobacter* genus isolates in the open access *Cronobacter* PubMLST database
82 (<http://www.pubmlst.org/cronobacter/>), and this species has been linked to several
83 fatal NEC and meningitis cases around the world [7-10]. *C. sakazakii* have been

84 isolated from prepared infant feeds associated with neonatal intensive care unit
85 (NICU) infections. Cases of necrotising enterocolitis (NEC), bacteraemia, and
86 meningitis have a 40-80% mortality rate, and 20% of the survivors develop serious

87 neurological disorders [11-14]. *C. sakazakii* distinct pathovars which are clonal
88 lineages, of particular clinical significance being clonal complex 4 (CC4) that contains

89 sequence type 4 (ST4), as well as ST12. These are strongly associated with invasive
90 meningitis and NEC cases, respectively [15]. One of the most studied NICU
91 outbreaks was in 1994 when 3 infants died from infections by *C. sakazakii* ST4
92 strains [14].

93

94 For organisms to establish a systemic infection they must adhere to the host cell,
95 translocate to the underlying tissues, and then disseminate throughout the body.

96 Therefore, the intestinal epithelium has an important role in protecting the body
97 against bacterial invasion. Once this layer loses its integrity, the invading organism
98 can infect the tissue beneath [16]. The ability of *C. sakazakii* to invade the intestinal

99 epithelium and brain endothelium is therefore a crucial step for its pathogenesis. It
100 was shown previously that *C. sakazakii* has the ability to adhere to epithelial and
101 endothelial cells *in vitro* [13, 17]. A study by Townsend et al. [18] used isolates from

102 the French outbreak in 1994, and showed that the *C. sakazakii* strains were able to
103 adhere and invade Caco-2 and rat brain capillary endothelial cells (rBCEC4) cell
104 lines. Moreover, the organism was able to persist and multiply within the human

105 macrophage U937 cell line [19]. Another study by Giri et al. [12] showed that food
106 and environmental strains of *C. sakazakii* have the ability to invade the HeLa subline

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

109

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

119

120 A number of virulence traits have been identified in *Cronobacter*, which may facilitate
121 the invasion and dissemination of the organism in the host. Franco et al. [21]
122 reported that the plasmid-borne *Cronobacter* plasminogen activator (Cpa) may
123 provide resistance to bactericidal activity of serum through cleaving complement
124 components C3 and C4b, and the activation of plasminogen and inactivation of α -
125 AP. In a study of over 100 *Cronobacter* genomes, *cpa* was found in *C. sakazakii* and
126 not *C. malonaticus* [23; <http://pubmlst.org/cronobacter/>], and therefore may contribute
127 to the higher clinical incidence of this species. It has also been reported that the
128 outer membrane protein A (OmpA) of *Cronobacter* spp. has a role in the colonisation
129 of the gastrointestinal tract (GIT) [21, 23]. Also, it was demonstrated that the outer
130 membrane proteins OmpA and OmpX were required for the basolateral invasion of
131 enterocyte-like human epithelial cells by *C. sakazakii* [23]. Singamsetty et al. [24]
132 demonstrated that the entry of *Cronobacter* spp. into HBMEC requires *ompA*
133 expression and depends on microtubule condensation in these cells. This might help
134 in the invasion of human intestinal cells and invasion of the brain endothelial cells to
135 cause meningitis [25]. Moreover, it was recently shown that *C. sakazakii* ST4 strain
136 767 was able to produce outer membrane vesicles (OMVs) that have the capacity to
137 increase the host's cell proliferation and stimulate a pro-inflammatory innate immune
138 response [26].

139

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

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

146

147 **2. Results**

148

149 *2.1. Invasion efficiencies of C. sakazakii clinical isolates to Caco-2 and HBMEC cell*

150 *lines*

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152 The invasion assay, using gentamicin protection to kill the extracellular bacteria, was
153 used to assess the ability of 10 *C. sakazakii* clinical isolates to invade the Caco-2
154 and HBMEC cell lines. With regard to the Caco-2 cell line, different invasion levels
155 were noted among these isolates, and strain 695 was the most significant ($P<0.05$).

156 The level of invasion by 695 was as high as *S. Enteritidis*, which was used as
157 positive control strain for the assay. Strains 20, 767, 1221, and 696 were moderate in
158 invasion, whereas strains 1240, 1242, 1249, 658 and 680 were low (Fig.1).

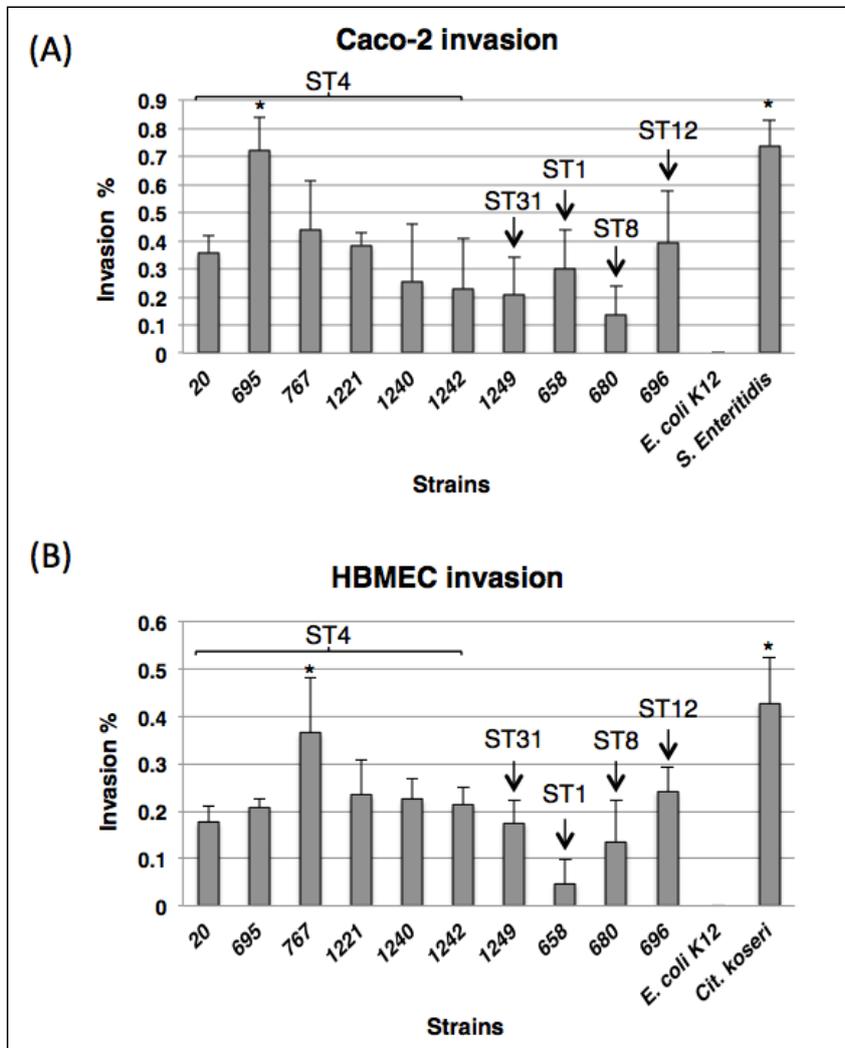
159 Regarding the HBMEC cell line invasion, strain 767 was the most significant ($P<0.01$)
160 being as high as *Cit. koseri*, the positive control. The other strains were moderate
161 except for strains 658 and 680, which were the lowest (Fig.1).

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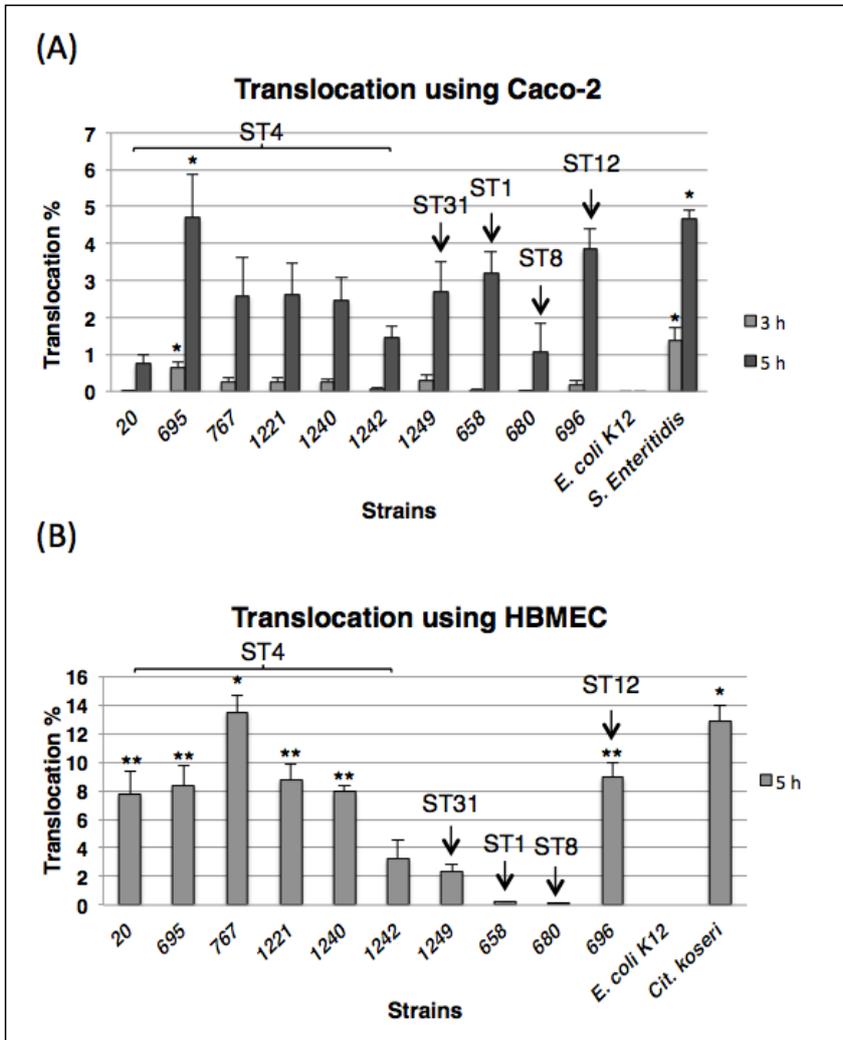
166 Fig.1. *C. sakazakii* invasion assay using Caco-2 (A) and HBMEC (B) cell lines over 3
 167 hours of incubation showing the differences in invasion levels among strains. The
 168 displayed data are the mean±standard deviation of invasion efficiency % of the initial
 169 inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks
 170 above the bars indicate statistically significant differences ($*P<0.05$; Kruskal-Wallis).
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175 2.2. Translocation of *C. sakazakii* clinical through Caco-2 and HBMEC polarised
176 monolayers

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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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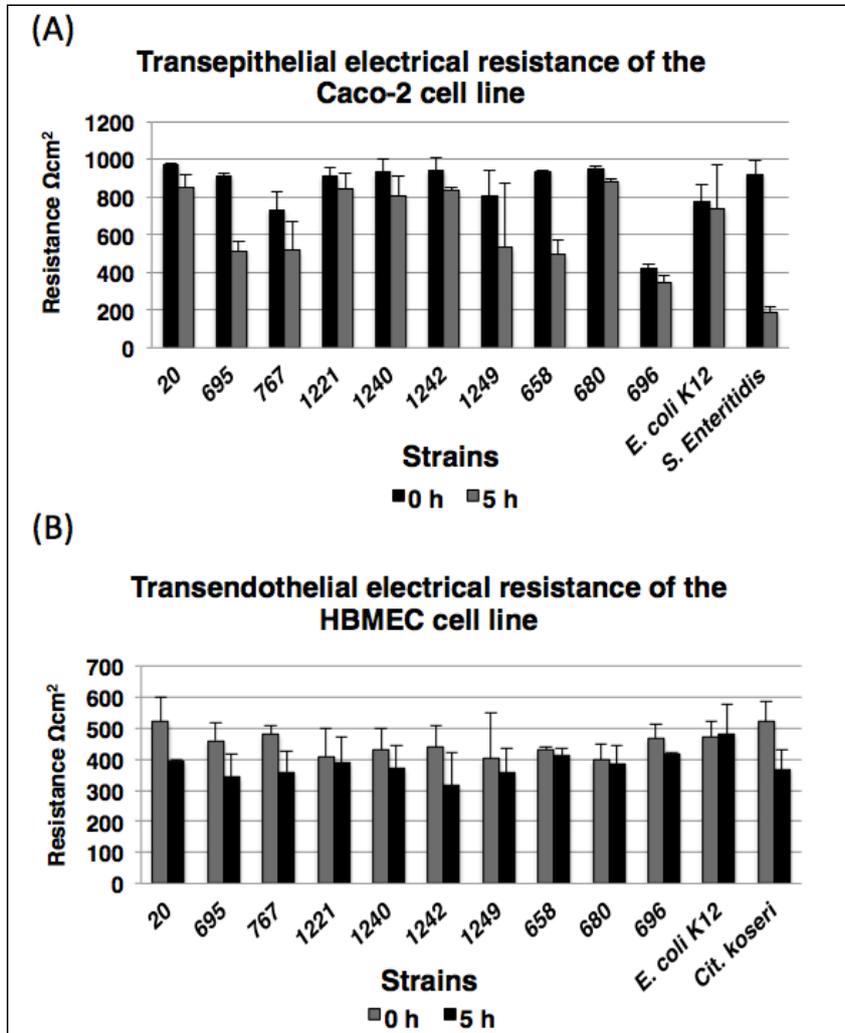


194 Fig.2. *C. sakazakii* translocation assay using Caco-2 (A) and HBMEC (B) cell lines
 195 over 5 hours of incubation showing the differences in translocation ability among
 196 strains. The displayed data are the mean±standard deviation of translocation
 197 efficiency % of the initial inoculum (10⁶ cfu/ml) of two independent experiments. The
 198 asterisks above the bars indicate statistically significant differences (**P*<0.001, **
 199 *P*<0.01; Kruskal-Wallis).
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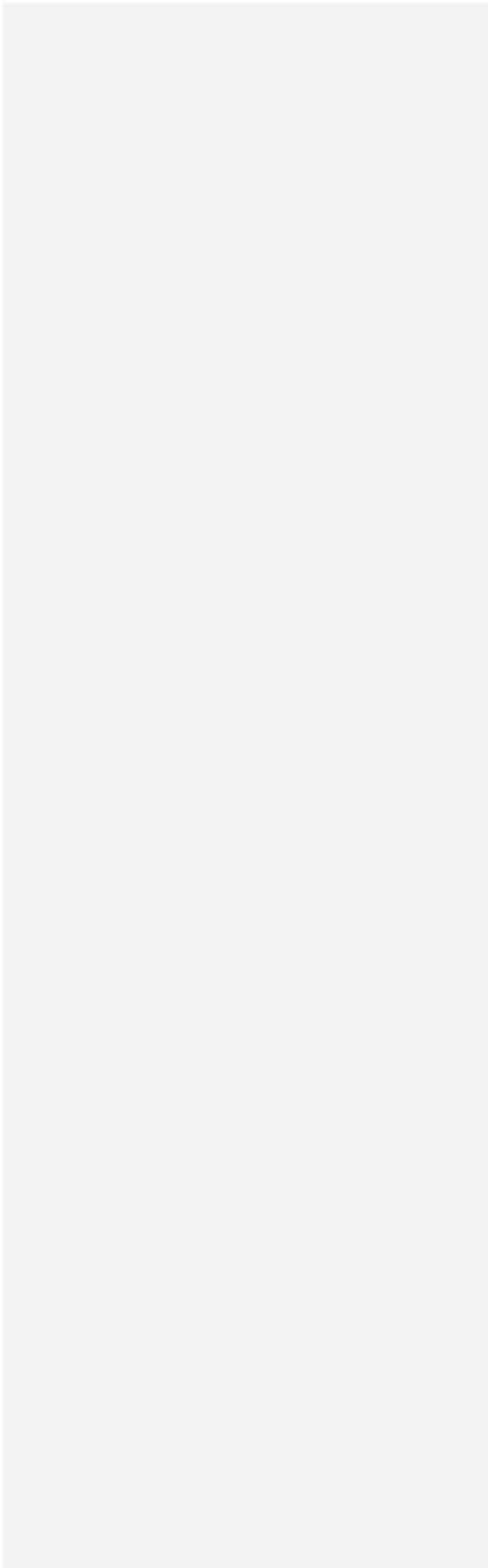
204 Fig.3. Transepithelial electrical resistance (TEER) of the Caco-2 cell line (A) and the
 205 transendothelial electrical resistance (TEER) of the HBMEC cell line (B) over 5 hours
 206 of incubation, showing changes in resistance over time.
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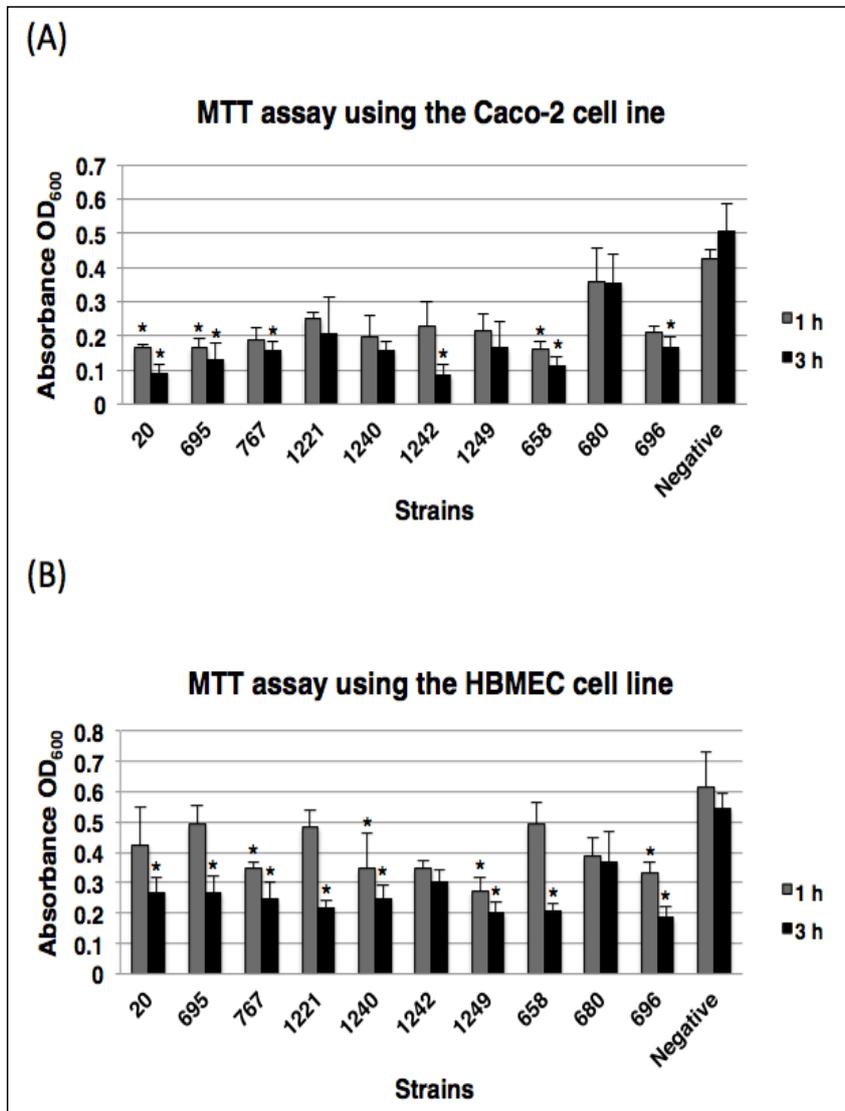
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2.3. *C. sakazakii* cytotoxic effect on the Caco-2 and HBMEC cell lines

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).





258 Fig.4. Cytotoxicity of *C. sakazakii* strains on Caco-2 (A) and HBMEC (B) up to 3
 259 hours of incubation. MTT reduction was used to measure the cytotoxicity levels of *C.*
 260 *sakazakii* strains where only the viable HBMEC cells are able to reduce MTT to its
 261 insoluble purple form formazan, the higher absorbance (OD₆₀₀) the higher in MTT
 262 reduction (low toxicity) and vice versa. The negative control used was uninfected
 263 cells treated using the same protocol with no bacteria added. The data presented in
 264 mean±standard error of mean of three independent experiments. The asterisks
 265 above the bars indicate statistically significant differences between the strains in this
 266 experiment (**P*<0.05; ANOVA).
 267

268 2.4. *C. sakazakii* survival within human macrophages U937

269

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

278

279 2.5. *C. sakazakii* survival within human microglial cells

280

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

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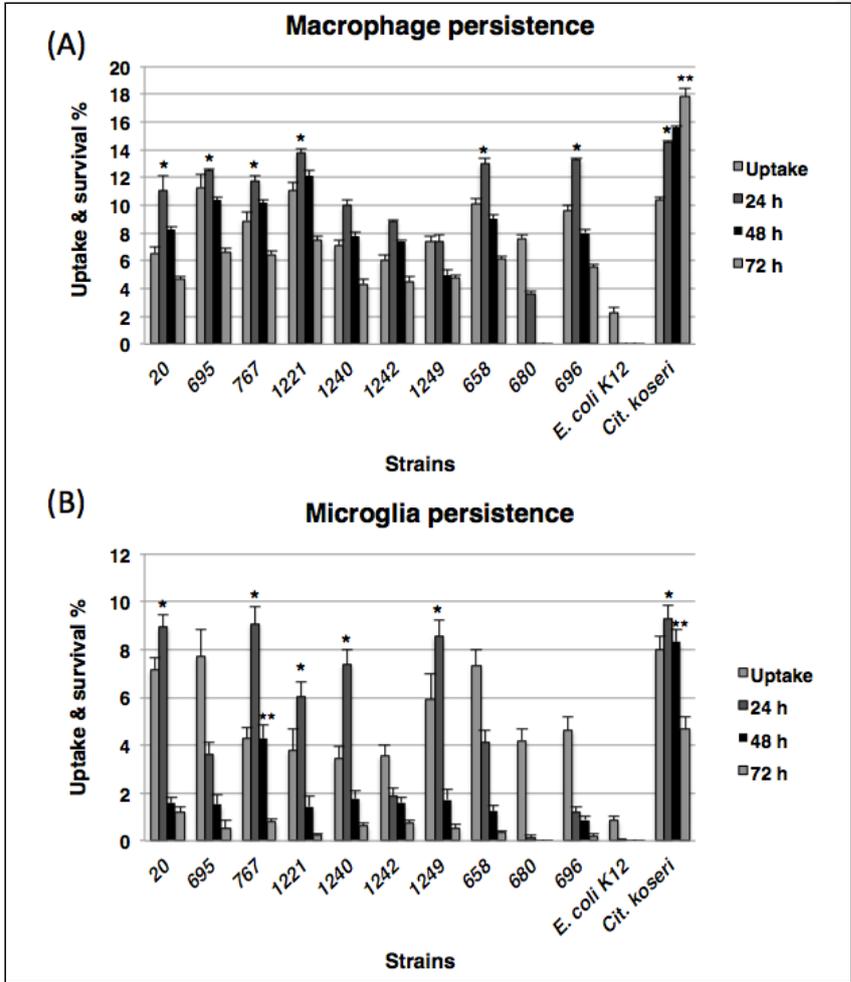
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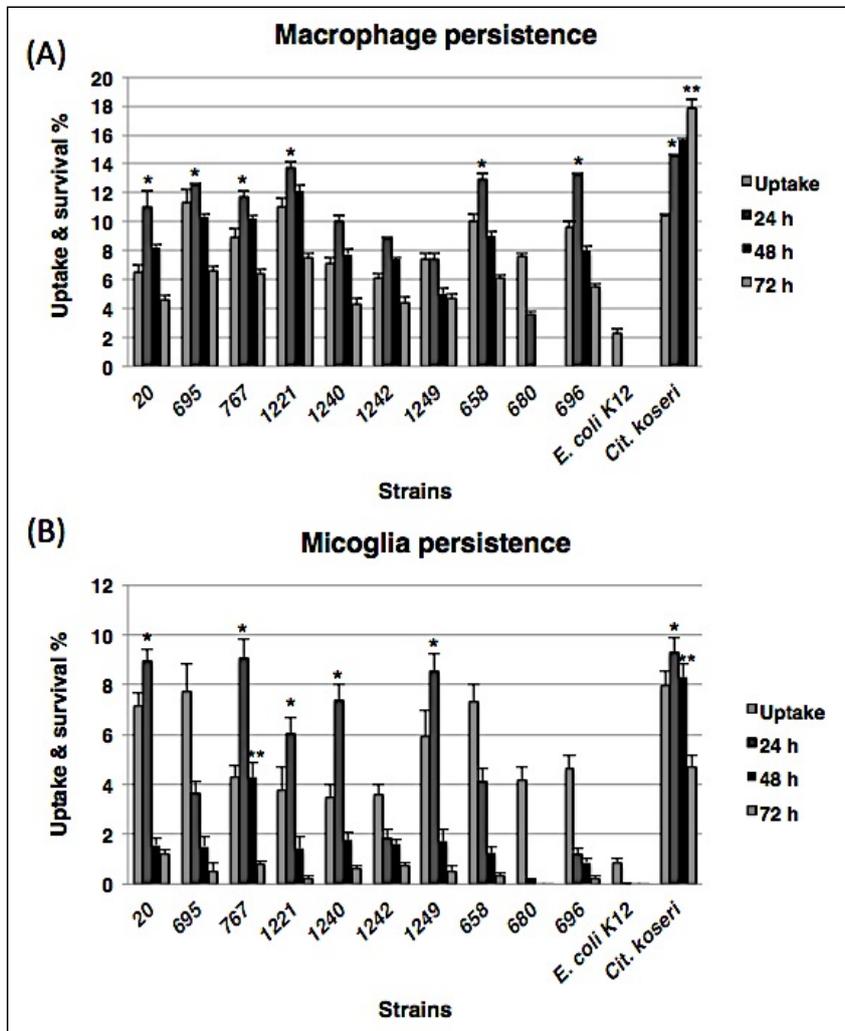
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346 Fig.5. *C. sakazakii* uptake and persistence assay using human macrophage (A) and
 347 human microglia (B) cell lines over 72 hours of incubation showing the differences in
 348 survival among strains. The displayed data are the mean±SEM for uptake and
 349 persistence efficiency % of the initial inoculum (10^5 cfu/ml) of three independent
 350 experiments. The asterisks above the bars indicate statistically significant differences
 351 (* P <0.05, ** P <0.001; ANOVA).
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357 2.6. *C. sakazakii* resistance to human serum

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359 All *C. sakazakii* strains in this experiment ($n=10$) showed up to 60% decrease in their
360 viable count at the first hour incubation with human serum (Fig.6). After the second
361 hour of incubation, it was noted that 9/10 strains showed an increase in their viable
362 numbers, whereas strain 680 showed a 30% reduction in its viability. The majority of
363 the strains were able to survive in human serum and increased by up to 4-fold in their
364 numbers after 3 hours of incubation. The exception was strain 680, which was serum
365 sensitive and its growth declined dramatically to 60% ($P<0.05$). Strains 20, 1242,
366 1249, and 696 were the highest in serum tolerance showing considerable elevated
367 growth rates (>200%) during the period of the assay.

368

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

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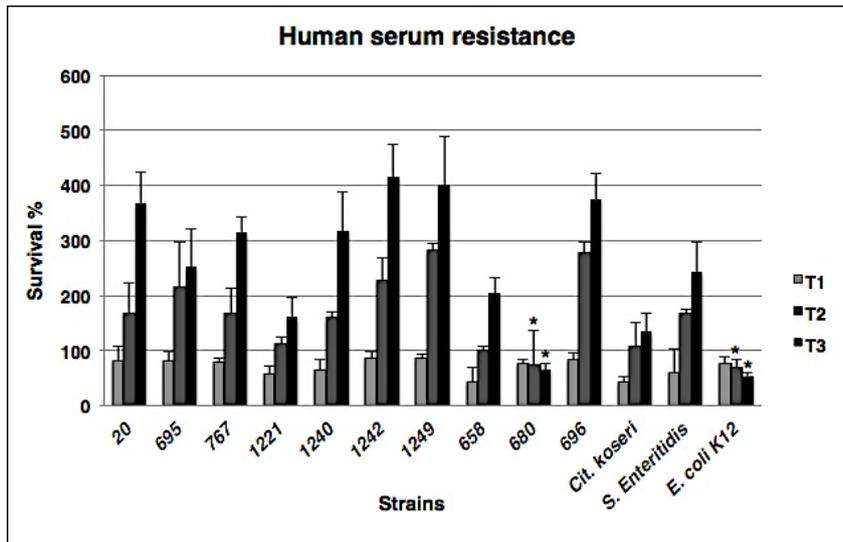
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397 Fig.6. Sensitivity of *C. sakazakii* to human serum over 3 hours of incubation showing
398 the difference in growth among strains overtime. Most of the strains showed
399 increases in their viable counts, and strains 6 (ST4), 680 (ST4), and *E. coli* K12
400 showed significantly declined values. The displayed data are the mean±standard
401 deviation of survival % (10^6 cfu/ml initial inoculum) of two independent experiments.
402 The asterisks above the bars indicate statistically significant differences (* $P < 0.05$;
403 Kruskal-Wallis).
404

405 3. Discussion

406
407 This study used a range of clinical isolates of *C. sakazakii*. These strains were
408 diverse with respect to their temporal and geographical sources, and all but one
409 strain were from patients with clinical presentations. The exception was *C. sakazakii*
410 658 (ATCC BAA-894) which had been isolated from the formula, that was not
411 intended for infants, used in the fatal NICU *C. sakazakii* outbreak at the University
412 of Tennessee [7]. The corresponding CSF isolate has not been deposited in any
413 international culture collection and therefore the PFGE indistinguishable strain
414 (ATCC code BAA-894) was used instead. This was also the first genome-sequenced
415 strain of *Cronobacter*, including the pESA3 plasmid which encodes for the *cpa* gene
416 [2].
417

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

427
428 Overall, the invasion results indicated that the majority (9/10) of the strains were able
429 to invade the cell lines and therefore potentially translocate towards the underlying
430 tissues and blood stream. Consequently, this could lead to the dissemination of the
431 organism around the body. To further investigate the translocation process further,
432 assays using the same human cell lines were applied. All strains were able to
433 translocate through the Caco-2 cell line especially strain 695 that displayed
434 significant high translocation ability ($P<0.01$), while strain 696 (ST12) was moderate
435 (Fig.2). These strains were able to cause high cytotoxicity levels to the Caco-2 cell
436 line, which could lead to increase the cell line permeability causing more bacterial
437 translocation (Fig.4). Strain 695 (ST4) accounted for a fatal neonatal NEC infection
438 while strain 696 (ST12) was previously linked to neonatal NECII infection [14]. The
439 invasion and translocation results correlate their virulence and their ability to cause
440 that disease.

441
442 There was a significant decrease in TEER when using the invasive and translocating
443 strains indicating a disruption in the tight junctions (Fig.3). This might explain the
444 mechanism of translocation for the strains where they can migrate in between the
445 cells by altering the tight junctions. This might trigger the onset of NEC, which could
446 lead to the spread of the bacteria in the blood stream. This clinical presentation of the
447 organism indicates the potential to reach the brain microvascular endothelium and
448 invades its cells. Therefore the translocation assay using the HBMEC cell line was
449 initiated to investigate whether the organism has the ability to translocate through
450 these cells or not. ST4 strains 20, 695, 1221, and 1240 in addition to 696 (ST12)
451 were high in translocation ($P<0.01$), and strain 767 (ST4) was the most significant in
452 translocation over 5 hours of incubation ($P<0.001$; Fig.2). *C. sakazakii* strains 1242
453 (ST4) and 1249 (ST31) were moderate in translocation, whereas 658 (ST1) and 680
454 (ST8) were the lowest. Strains 695 (ST4), 767 (ST4), and 696 (ST12), which showed

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

464
465 The translocated strains might need a longer infection period to accomplish the
466 translocation process, as the translocation was noted 5 hours post infection, while in
467 Caco-2 it was after 1 hour with some strains. Moreover, they could use different
468 mechanisms of invasion to overcome this barrier that is a part of the BBB. One of the
469 possible mechanisms that might assist the strains to translocate is the bacterial
470 cytotoxicity that leads to initiate cell death in the cell line via apoptosis or necrosis.
471 Moreover, the cytokines released by the cells might play a role in the same process
472 by making the cell line permeable. High levels of nitric oxide (NO) are a potential
473 factor that could contribute in the permeability of the cell line. Also, OMVs might
474 contribute in this process by triggering the host proinflammatory response leading to
475 the secretion of some inflammatory mediators, including cytokines and NO, that in
476 turn could cause cell line permeability [26]. The translocation of these strains
477 indicated their potential to pass through towards the brain tissues triggering the host
478 response, which could result in brain inflammation and tissue damage.

479
480 In a previously published study by Giri et al. [12], non-clinical *C. sakazakii* isolates
481 were examined and showed their ability to invade and translocate through the Caco-
482 2 and HBMEC cell lines intracellularly. In this research, the same cell lines were used
483 to compare the results of the clinical strains in our study and the non-clinical strains
484 from the previous research by Giri et al. [12] with regard to the capacity and
485 mechanism of translocation. However, the results~~We found that~~ of the capacity of
486 translocation~~translocation ability~~ of *C. sakazakii* clinical isolates ~~that were obtained~~
487 ~~by our research were was~~ more than 10 times higher than the previous study using
488 the same cell lines. Additionally, our results ~~showed~~ suggested a different
489 mechanism of translocation, which is~~their capacity of~~ paracellular translocation
490 through those cell lines. All of the strains were from clinical sources and were linked
491 to severe and fatal neonatal cases.

492

493 The survival studies were conducted to investigate the ability of the test strains to
494 survive and multiply within macrophages and microglia which are both types of
495 immune cells. *C. sakazakii* strains used in the survival experiments showed the
496 ability to persist within human macrophages cell line U937 for up to 72 hours of
497 incubation. Moreover, ST4 strains 20, 767, and 1221 in addition to 658 (ST1) were
498 able to survive and multiply significantly ($P < 0.001$). The survival results were
499 comparable to the results obtained previously by Townsend et al. [18]. Strains 767
500 (ST4) and 696 (ST12) showed similar persistence and multiplication levels as the
501 ones published in that research. However, Townsend et al. [18] reported that strain
502 695 (ST4) was able to survive and could not multiply within macrophages. These
503 results are in contrast of the ones obtained here as strain 695 (ST4) showed the
504 ability to survive and multiply in U937 cells. Our results correlate the virulence
505 potential of the strain, and the invasion and translocation profiles, as it was an
506 invasive strain to both of the Caco-2 and HBMEC cell lines. Moreover, it is a clinical
507 strain that was linked to a fatal NEC infection [14]. This suggests that this strain was
508 able to establish a successful infection and has the virulence traits to avoid
509 phagocytic killing.

510

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

524

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

529 to survive within human microglial cells. It was shown in this research that the
530 majority of *C. sakazakii* strains were able to persist in human microglia as
531 represented by the HMGc cell line for 72 hours. Moreover, ST4 strains 20, 767,
532 1221, and 1240 in addition to 1249 multiplied significantly in this cell line ($P<0.001$).
533 Although these strains showed the ability to multiply within microglia, they
534 nevertheless showed declined levels of persistence afterwards. The ability of the
535 bacteria to reproduce intracellularly within these phagocytic cells demonstrates their
536 virulence potential to withstand the bactericidal activity of microglia and evade the
537 host immune response.

538

539 Invasive microorganisms have protective mechanisms against serum-mediated
540 killing. Bacterial structures including outer membrane proteins and proteases were
541 identified for their roles to avoid this bactericidal action [34-36]. Franco et al. [21]
542 showed that the Cpa is a plasminogen activator that plays an essential role in
543 *Cronobacter* serum resistance. Nine of the ten *C. sakazakii* strains in this research
544 were regarded as serum resistant being able to replicate in human serum and
545 appeared to be completely refractory to serum killing. Whereas *C. sakazakii* strain
546 680 was serum sensitive and showed significant reduction in viability ($P<0.05$; Fig 6).

547

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

559

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

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

584

585 **4. Materials and methods**

586

587 *4.1. Bacterial strains*

588

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

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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

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

611

612 4.2. Cell culture

613

614 Human colonic carcinoma epithelial cells (Caco-2) passages 17 to 45 acquired from
615 the European Collection of Cell Cultures (ECACC #86010202) and human brain
616 microvascular endothelial cells passages 2 to 25 (HBMEC; ref. #HMG030 Inoprot,
617 Spain). Macrophage cell line (U937) passage 12 was obtained from American Type
618 Culture Collection (ATCC; #CRL-1593.2), and human microglial cell line passage 3
619 was obtained from Innoprot Technologies (Ref.# P10354). All experiments were
620 applied at consistent conditions of time, temperature, cell line passage, mammalian
621 cells concentration, and bacterial suspension. *Salmonella* Enteritidis strain NTU 358
622 was used as positive control for Caco-2 cell line, while *Citrobacter koseri* strain NTU
623 48 was the positive control for HBMEC, macrophages, and microglial cell lines.
624 *Escherichia coli* K12 strain NTU 1230 was the negative control for all cell lines.

625

626 4.3. Bacterial invasion of mammalian cells

627

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

632 grown in Dulbecco's modified eagle medium (DMEM) with 10% (v/v) FCS and 1%
633 (v/v) penicillin-streptomycin (Sigma Aldrich, UK). Mammalian cells then were seeded
634 at 4×10^4 cell/well in growth medium for 48 hours in 5% CO₂ at 37°C to achieve a
635 confluent monolayer. *C. sakazakii* strains were grown in LB broth for overnight at
636 37°C. The suspension then was added to the wells at MOI 100, and incubated in 5%
637 CO₂ at 37°C for 2 hours. The wells were then washed using PBS (Sigma Aldrich,
638 UK). Then, 0.5 ml of infection medium supplied with 125 µg/ml (v/v) of gentamicin
639 (Sigma Aldrich, UK) was added and incubated in 5% CO₂ at 37°C for 1 additional
640 hour. The wells were then washed with PBS (Sigma Aldrich, UK) before lysing by 1%
641 (v/v) Triton X-100 (Fisher Scientific, UK), and plated on TSA at 37°C for overnight
642 incubation after serial dilution to obtain viable count. Data are presented as the
643 percentage efficiency of invasion.

644

645 4.4. Translocation assay using Caco-2 cell line

646

647 Translocation assay was performed as previously described [12, 38]. However, 0.8
648 ml of growth medium was added to the basolateral chamber of Millicell-24 cell culture
649 plate (Millipore, UK). Caco-2 cells, at a concentration of 4×10^4 cell/well in 0.4 ml/well
650 of Minimum Essential Medium (MEM) supplied with 10% (v/v) foetal calf serum
651 (FCS), 1% (v/v) non-essential amino acid (NEAA), and 1% (v/v) penicillin-
652 streptomycin (Sigma Aldrich, UK), were seeded onto a 3 µm pore polycarbonate
653 transwell membrane in the apical chamber of the tissue culture plate and incubated
654 in 5% CO₂ at 37°C. The medium in the apical and the basolateral chambers was
655 changed every 3 days. Millicell ERS-2 Volt-Ohm Meter (Millipore, UK) was used to
656 measure the TEER. The Caco-2 cell line required up to 21 days to form intact
657 polarised monolayers with TEER 300-850 Ωcm² [12, 38, 39]. On the day of the
658 assay, the medium in the basolateral chamber was replaced with infection medium.
659 The medium in the apical chamber was removed, and the membrane was washed
660 using 0.4 ml of PBS (Sigma Aldrich, UK). Bacterial suspensions was prepared was
661 added at MOI 100 to the apical chamber. At each time point of incubation, the
662 basolateral chamber was sampled for viable count after serial dilution and inoculation
663 on TSA. The TEER was measured at each time point. Data are presented as the
664 percentage efficiency of translocation.

665

666 4.5. Translocation assay using HBMEC cell line

667

668 This assay was carried out using the protocol described previously [12, 40, 41]. The
669 basolateral chambers of the 24-well plate were filled with 0.510 ml/well of Dulbecco's
670 modified eagle medium (DMEM) with 10% (v/v) FCS and 1% (v/v) penicillin-
671 streptomycin (Sigma Aldrich, UK). Cells, with a concentration of 4×10^4 cell/well in
672 0.375 ml/well of previous medium, were seeded onto the apical part of collagen-
673 coated polytetrafluoroethylene (PTFE) membrane with a pore size of 0.4 μm
674 (Transwell-COL; Corning, USA), and incubated in 5% CO_2 at 37°C. The medium in
675 the apical chamber was changed every 3 days. The transendothelial electrical
676 resistance (TEER) was measured using Millicell ERS-2 Volt-Ohm Meter (Millipore,
677 UK). According to electrical resistance measurements, HBMEC cell line required 5 to
678 8 days to form intact polarised monolayers with TEER 300-600 Ωcm^{-2} [12, 42]. Prior
679 to infecting the cell line, the filter was washed by 0.375 ml/well PBS (Sigma Aldrich,
680 UK), and the medium in the basolateral part was replaced by infection medium. The
681 cell line was infected by 0.375 ml per well bacterial suspension with MOI of 100. The
682 basolateral chamber was sampled and serially diluted and then plated for viable
683 count on TSA at 37°C before replacing with a fresh infection medium. Moreover, the
684 TEER of the monolayers was measured at each time point. Data are presented as
685 the percentage efficiency of translocation.

686

687 4.6. *C. sakazakii* cytotoxic effect on the Caco-2 and HBMEC cell lines

688

689 The ability of *C. sakazakii* to induce cytotoxicity was assessed using the colorimetric
690 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay. This assay
691 is based on the reduction of MTT (Sigma Aldrich, UK) by viable cells to its insoluble
692 form formazan, which has a purple colour. This experiment was applied as described
693 previously [43, 44]. Briefly, bacterial suspensions at a concentration of 4×10^6 cfu/well
694 (MOI 100) were added to confluent monolayers of the Caco-2 and HBMEC cell lines.
695 The plates were then incubated in 5% CO_2 at 37°C for 1 and 3 hours. A volume of 50
696 μl of MTT was added per 500 μl of culture medium. Next, the medium containing
697 MTT was removed and formazan was solubilised in dimethyl sulfoxide (DMSO;
698 Fisher Scientific, UK). The absorbance was measured at 600_{nm} after 3 hours. The
699 negative control for the assay consisted of uninfected cells.

700

701 4.7. *C. sakazakii* persistence in human macrophages

702

703 As previously given by Townsend et al. [19] with slight modifications, macrophages
704 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 4×10^4 cell/well and incubated in 5% CO₂ 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% CO₂ 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% CO₂
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 cm³ tissue culture flask. The cells then were seeded into four 24-well plates at a
728 concentration of 4×10^4 cell/well and incubated in 5% CO₂ 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% CO₂ 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% CO₂ 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

742 The sensitivity of *C. sakazakii* strains to active human serum was conducted as
743 described previously by Hughes et al. [46] with slight modification. Bacterial cultures
744 were grown overnight in LB at 37°C with shaking at 200 rpm then centrifuged for 10
745 minutes at 1300 rpm (Mikro 200-Hettik). The pellet then was re-suspended to 10⁶
746 cfu/ml in 5 ml of phosphate buffered saline (PBS; Sigma Aldrich, UK). A volume of
747 0.5 ml of the suspension was added into 1.5 ml of undiluted active human serum
748 (Sigma Aldrich, UK). The samples were loaded into a 24-well plate and incubated at
749 37°C. Viable counts were obtained at 4 different time points. Data are displayed in
750 percentage of percent survival of inoculum.

751

752 4.10. PCR probing for *cpa* gene and BLAST genome search

753

754 Plasmid DNA extraction was carried out according to the manufacturer's instructions
755 using QIAprep Spin Miniprep Kit (Qiagen, UK). PCR primers were designed to target
756 *cpa* loci on the large *C. sakazakii* plasmid pESA3 plasmid. GoTaq® DNA Polymerase
757 kit (Promega, UK) was used for all PCR reactions' preparation. Primers design, and
758 the PCRs were all conducted according to Franco et al. [25]. All reactions started
759 with 3 minutes at 94°C and denaturation step at 94°C for 30 seconds, followed by 25
760 cycles of 30 s at 56 °C and 30 s at 72 °C. The final extension period was 10 min at
761 72 °C. Primer pair *cpafw*, 5'-GACAACCCTGAGTTCTGGTAAC, and *cparv*, 5'-
762 ATGCGTATTTCTGCTGGTAA, targets a 306 bp region. Moreover, BLAST genome
763 search was applied to strains using the sequence of *cpa* gene to confirm its
764 presence. The BLAST search was at <http://www.pubmlst.org/cronobacter>.

765

766 4.11. Statistical analysis

767

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

778 statistical analysis software was used to perform the analysis (IBM SPSS version
779 22.0, Chicago, IL, USA).

780

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786

787

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789

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