ORIGINAL ARTICLE

Protein Expression of Human Brain Microvascular Endothelial Cells in Response to Meningitic C. Sakazakii: in Vitro and in Silico Analyses

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ABSTRACT

Background: Despite the confirmed association between infant meningitis and necrotizing enterocolitis and C. sakazakii lettile known about the pathogenic interaction of C. sakazakii with host cells.
Aim: To characterize functional and regulatory proteins that are differentially expressed in Human brain microvascular endothelial cells (HBMEC) in response to meningitic *C. Sakazakii*
Method: Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) analysis was performed to the protein extracted from HBMEC in response to meningitic *C. Sakazakii* in serial time course. Bioinformatics analysis was carried out using SCIEX OneOmics cloud processing software and Advaita's iPathway**.**
Results: In response to the exposure, the LC-MS/MS analysis indicated different changes in 57 proteins: nucleic acid binding proteins, transporter proteins and structural molecule activity proteins. 1, 9 and 29 pathways were significantly affected after 60, 90 and 120 minutes, respectively. In addition, 179, 242 and 490 gene ontology components were found to be significantly enriched. Interestingly, the tight junction pathway , which is most important component regulating to brain damage, was triggered at 120 minutes post-infection with the C. sakazakii meningitic strain.
Conclusion: We conclude that the data presented in this study may facilitate a better understanding of this bacterium, which can cause irreparable damage to a new-born baby's brain. This study open up many new avenues into the research concerning this pathogenicity.
Keywords: Proteomic; HBMEC cells; C. sakazakii; Meningitis

INTRODUCTION

Cronobacter is an emerging pathogen of concern that is associated with severe and often fatal cases of infant meningitis and necrotising enterocolitis. Although a great deal of research has been conducted over the past decade to shed light on the virulence of *Cronobacter spp.* and elucidate their mechanisms of pathogenicity, survival and genetics, there are still many hidden traits to be revealed[1]. Therefore, *in vitro* studies have continued to investigate these phenomena and can potentially lead to better control of this pathogen and minimise its infections in infants, as well as the elderly and other immunocompromised people[1].

Measurement of protein expression has been detected in *Cronobacter spp.* using proteomic tools to explain the adaptation mechanisms of osmotically stressed cells[2]. Virulence of *C. turicensis was investigated by focusing on* proteins characterisation and leading to conclude the involved molecular mechanisms[3].

Likewise, another study by histopathological analysis the potential virulence factors at the proteomic level detected[4]. All of the proteomics studies so far have successfully evaluated *Cronobacter spp.* at the protein level; however, the protein expression of infected host cells is a controversial topic that needs to be studied more[5]. Understanding how cells behave in the presence of a pathogen may provide key answers such as signaling during adhesion or a variety of other stressful responses during conditions[6]. Hence, the identification of pathogenic mechanisms is mainly based on the neat description of molecular process and the detailed protein expression profile .Thus, our study aims to investigate the protein expression modifications by proteomic analysis of HBMEC cells' response to meningitic C. sakazakii strain 767 to generate new insight into its pathogenetic mechanism. We opt to determine whole protein profiles of infected HBMEC cells with C. sakazakii strain 767 by comparing them to whole protein profiles of uninfected cells and evaluating whether the important proteins are up or down regulated over the exposure time. Further, differentially expressed proteins were classified based on the protein ontology to help us to analyse and understand functional and biological information about the identified proteins.

MATERIAL AND METHODS

Bacterial strain , cell culture and strain exposure: Convenient laboratory practices were monitored for microbes, chemicals, cell culture and operating laboratory devices. The strain was isolated from an eruption of *Cronobacter* meningitis infection[7]. Clinical isolate was selected based on the phenotype analysis and virulence assays of these strains and whole genome sequence availability[8].
Cell culture: *sakazakii* strain 767 was added to confluent monolayers of HBMEC cells ($1x10^6$) and incubated for 60, 90 or 120 minutes. Cell lysis, centrifugation, alkylation and reduction and trypsinisation were performed.
Liquid chromatography with tandem mass spectrometry (LC-MS/MS): Samples were concentrated to dryness in a Speed Vac set for aqueous solution evaporation (approximately 30–40 minutes). The samples were re-suspended and moved into a new LC vial (suspension solution: 20 µL acetonitrile 5% and formic acid 0.1%). Samples were chromatographically separated based on their affinity to a 20 cm C18 column using an

Eksigent nanoLC 425 system operating at microflow (5 uL) over a 120-minute gradient.

The preceding MS analysis used a SCIEX 6600 TripleTOF™ with either a data-dependent acquisition (DDA) method of a sample pool to generate a spectral library or with a SWATH™ data-independent acquisition (DIA) method on each sample separately for a reproducible quantitation of proteins. The spectral library was aligned to spiked iniRT Standard (Biognosys, Switzerland) that of the samples using PeakView 2.0 (SCIEX).All data were then uploaded to the Cloud computing Basespace software in the Oneomics platform for a calculated quantitative comparison of protein expression against the DIA data available at data-independent acquisition (DDA) library.

I n silico analysis: First, the ontology information for each differentially expressed protein were imported from the UniProt website through the accession numbers. This information used in the present experiment to generate ontology plots. The ontology connection filter of 75% is used to show only those ontology categories with the minimum number of differentially expressed proteins. The 75% protein confidence filter was chosen for a 120 minutes' exposure of HBMEC cells to strain 767 versus control (unexposed cells).

The data were next analysed in the context of pathways obtained from the KEGG database and gene ontologies from the Gene Ontology Consortium database.

Advaita's iPathway was applied to analyse the significantly affected pathways and related gene panel. It also provides details about role, type, function, position and interactions of each involved gene.

The protein fold changes could be visualised across the study as a heat map for specific proteins. iPathwayGuide is an additional application used in this study to link changes to the gene level.

RESULTS

Protein fold changes: A total of 2500 protein fold changes were determined by MS analysis used a SCIEX 6600 TripleTOF™. The heat map indicated different changes in 57 proteins that are summarized in table 1 with detailed proteins functions. The table present also differential level of expression in comparison with untreated cell after different exposure time and comparative study with other previous reports for each protein (Fig1, Table 1).

Protein ontology: There are three different types of ontology that can be viewed in Fig 2 namely (a) biological processes, (b) cellular components and (c) molecular functions. The majority of the protein ontology was upregulated, except for the biological processes of the viral process, gene expression and RNA metabolism, cellular components of cytosol, cytoplasm and the nucleus and the molecular functions of metal ion/RNA binding and structuring of ribosome (Fig. 2a, 2b, 2c).

Results showed that the metabolic process was the most prevalent biological process, representing around 30% (of the 57 proteins that in Fig 1) proteins altered in response to exposure strain 767 for 120 minutes.

the extracellular vesicular exosome was the most frequent cellular component, representing more than 50 proteins that were differentially expressed in response to the C. sakazakii meningitis isolate in comparison to unexposed HBMEC cells. On classifying differentially expressed proteins based on their molecular function revealed the overall representation of protein and nucleic acid binding proteins, transporter proteins and structural molecule activity proteins (**Fig 2.C**).

In the figure 2 we illustrates the three aspects of protein ontology, namely (A) biological processes, (B) cellular components and (C) molecular functions. The colored parts represent an estimate of the fold change in associated protein expressions. Red indicates up regulation and blue indicates down regulation. A white color indicates a neutral response with relatively equal evidence for both up- and down regulated proteins.

Gene ontology: In our study 34, 118 and 180 genes out of a total of 2500 genes were found to be differentially expressed in three serial time courses of 60, 90 and 120 minutes, respectively (table 2). It was found that 1, 9 and 29 pathways were significantly affected after 60, 90 and 120 minutes, respectively. In addition, 179, 242 and 490 gene ontology components were found to be significantly enriched in response to 60, 90 and 120 minutes exposure to the meningitic C. sakazakii strain. Table 2 shows results for three different time courses of exposure to the C. Sakazakii meningitis strain in relation to pathways and gene ontologies.

Functional ontologies based on Enrichment analysis: As shown in Fig 3, according to iPathway Guide application, the top pathways most affected by infection were the tight junction pathway (fig 3.a) and the apoptotic process pathway (fig 3.b). The tight junction pathway was triggered at 120 minutes post-infection with the *C. sakazakii* meningitic strain at the gene level as well (fig 3.b and 3.c).

Computed genes perturbation is described in diagrams. The box plot describe genes negative perturbation (blue), positive perturbation for tight junction (3.a) and apoptotic (3.b) pathways. The bar plot present gene involved the pathway ranked based on their absolute perturbation (positive: red, negative: blue) in tight junction (3.c) and apoptotic (3.d) pathways. The box represents the first quintile, the median and the third quintile, while the outliers are represented by circles.

Table 1. Differentially expressed proteins of C. Sakazakii 767 in HBMEC cell line.

Accession no.	Protein Name	Protein function description	Protein expression differential level after different exposure time *				Reference
			60 min	90 min	120 min	Trend	
P09211	Glutathione S-transferase P	Protecting cells as a free radical scavenger along with glutathione	–0.589	–0.622	–1.069	Down	[20]
P15531	Nucleoside diphosphate kinase A	Major role in the synthesis of nucleoside triphosphates	–0.577	–0.445	–1.062	Down	
P19623	Spermidine synthase	Plays a critical role in the movement of the gatekeeping loop on binding substrates	–0.412	–0.831	–0.884	Down	[21]
P69905	Haemoglobin subunit alpha	Involved in oxygen transport from the lung to various peripheral tissues	3.538	2.998	1.505	Down	
Q15907	Ras-related protein Rab-11B	Promotes autophagic flux, establishing the constitutive and cAMP; induces proapoptotic response	0.358	0.463	0.596	Up	[22]
P07602	Prosaposin	Modulates human invariant natural killer T cells' self-reactivity	–0.616	–0.598	0.612	Up	[23]
P21926	CD9 antigen	Plays an important role in the redistribution of peptidase activity fromthe cell surface to outer microenvironments by the interaction with CD10	0.202	0.418	0.654	Up	[24]
P07437	Tubulin beta chain	Tubulin is the major constituent of microtubules	–0.414	0.43	0.44	Up	
P68371	Tubulin beta-4B chain	Tubulin is the major constituent of microtubules	0.251	0.367	0.368	Up	
P80723	Brain acid soluble protein 1	Transcriptional regulation during differentiation	–2.904	0.48	1.489	Up	[10]
P27824	Calnexin	May act in assisting protein assembly	0.376	1.291	1.976	Up	
P06132	Uroporphyrinogen decarboxylase	Catalyses the decarboxylation of four acetate groups of uroporphyrinogen-III	–0.635	–0.772	–0.674	Down & up	
P61916	Epididymal secretory protein E1	Plays a role in the egress of cholesterol from the endosomal/lysosomal compartment	–0.464	–0.508	–0.29	Down & up	
P13987	CD59 glycoprotein	Associated with chronic haemolysis and childhood relapsing immune-mediated polyneuropathy	0.486	0.424	0.936	Down & up	[25]
P35613	Basigin	Targets the monocarboxylate transporters and plays a role in neural network formation	0.643	0.527	0.848	Down & up	
P07355	Annexin A2	Plays a role in cellular signal transduction, inflammation, growth and differentiation	0.608	0.371	1.543	Down & up	
P50995	Annexin A11	Required for midbody formation and completion of the terminal phase of cytokinesis	0.581	0.441	0.995	Down & up	[7]
P29401	Transketolase	Regulates cell growth and survival through glucose utilisation, purine synthesis, and RNA and DNA synthesis	–0.622	–0.668	–0.402	Down & up	

***The value exhibit the protein expression changes across the negative control (untreated cells**

Fig 1. A heat map of differentially expressed proteins from HBMEC cells. It identifies that 57 out of 2500 proteins were selected as differentially expressed proteins at a 75% confidence level. Colours within the heat map range from light blue (downregulated) to dark red (most upregulated), illustrating the relative abundance of each protein within a particular sample. The protein name has been aligned within the heat map according to the name in the UniProt database.

Fig 1

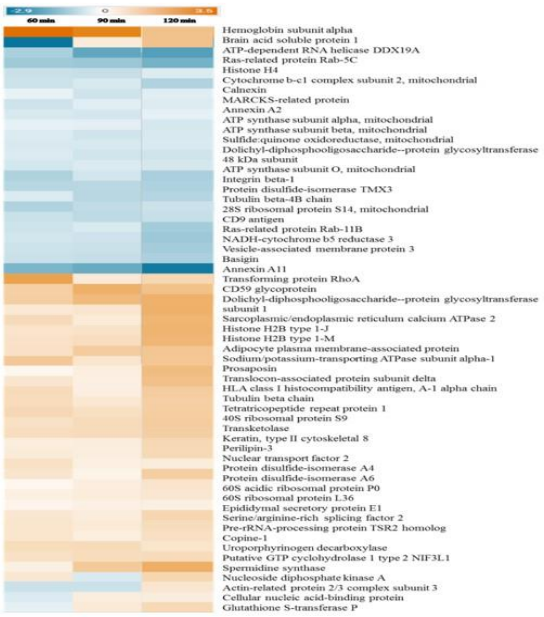


Fig. 2 : Protein ontology of expressed proteins in HBMEC cells exposed to *C. Sakazakii*

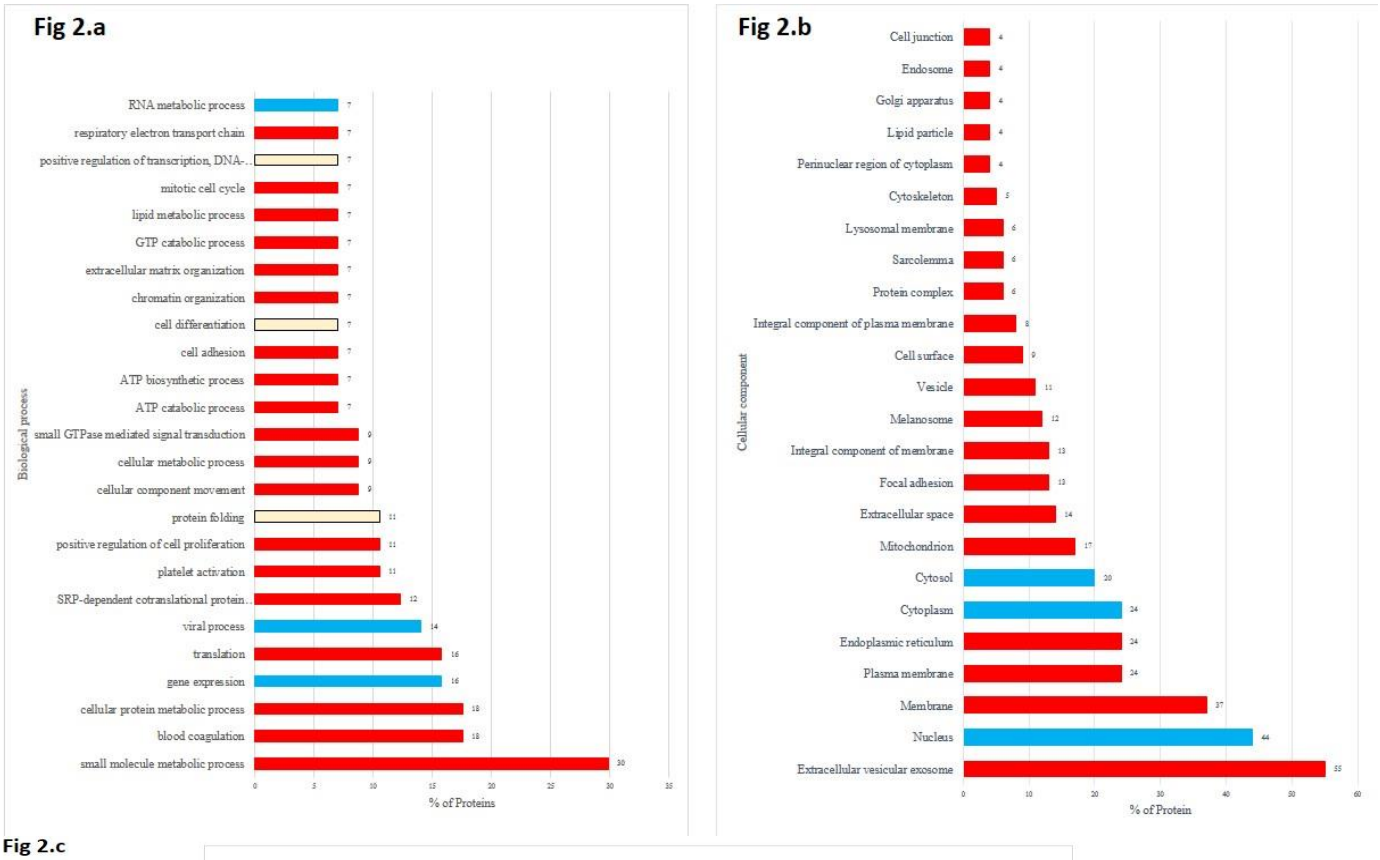


Fig 2.c

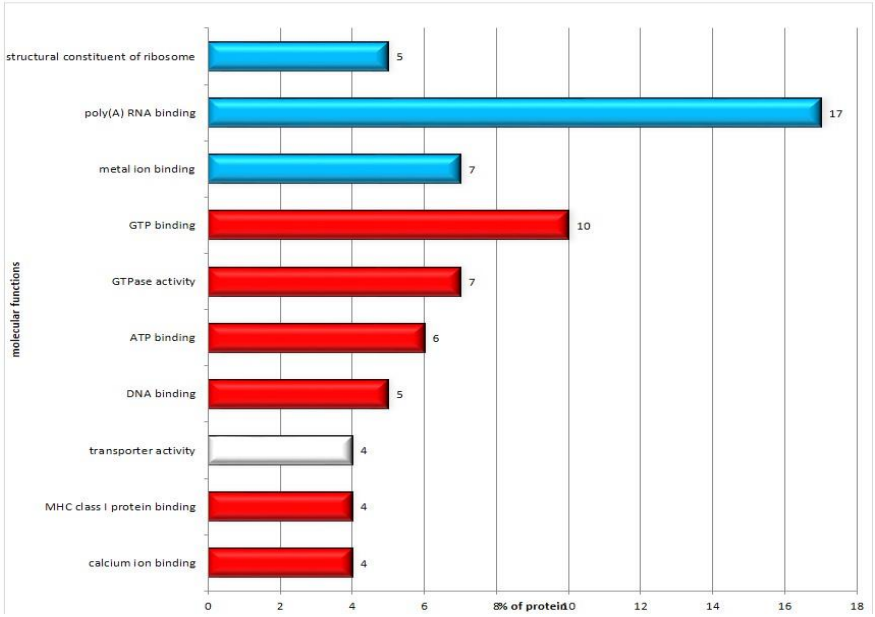
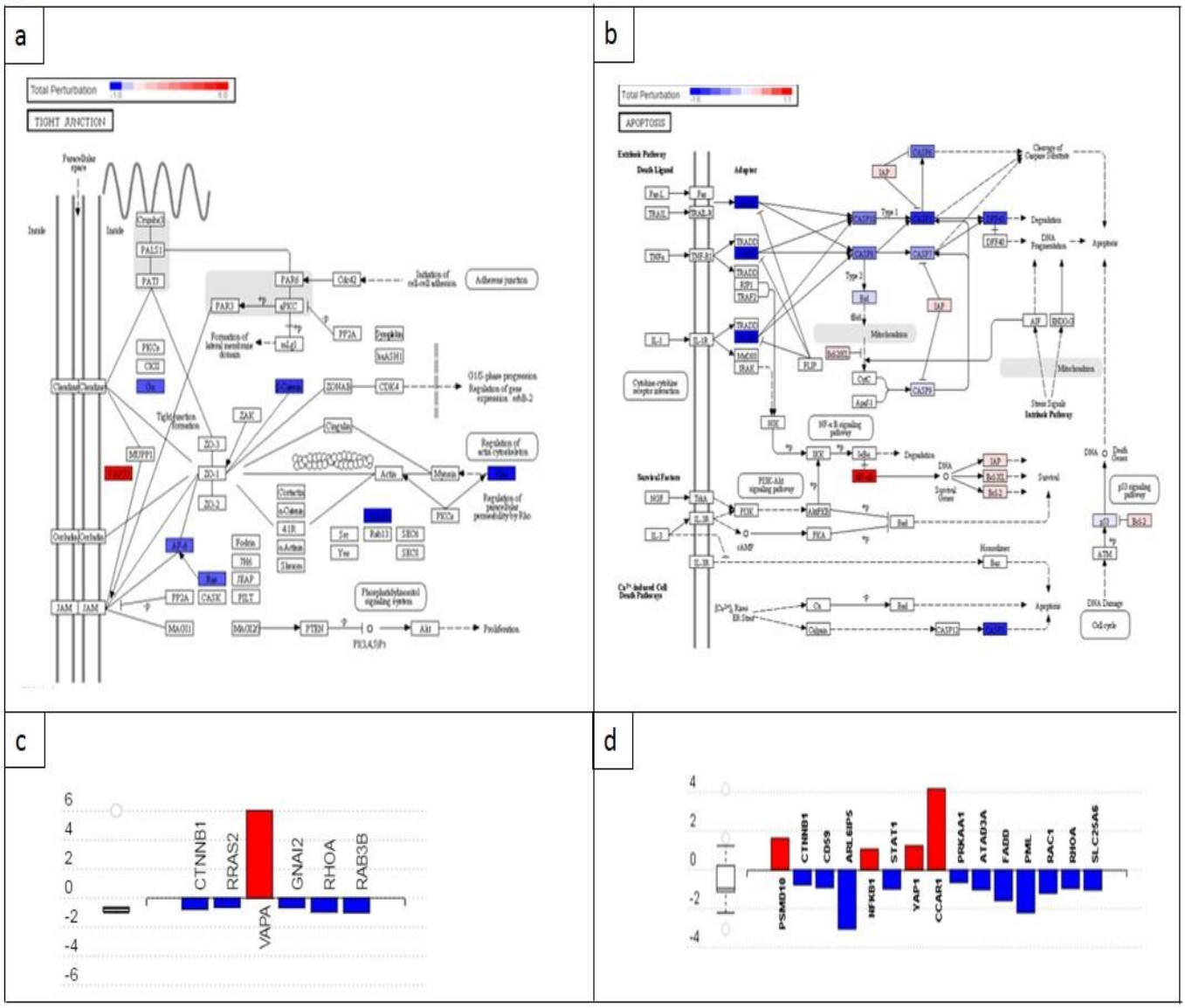


Table 2: Demonstration of the gene ontology of HBMEC cell after *C. sakazakii* 767 exposure

Time exposure	Pathways	Gene ontology
60 min	1	179
90 min	9	242
120 min	29	490

Fig 3. Tight junction and apoptotic pathways and gene perturbation.



DISCUSSIONS

This study reflects about on the host response in HBMEC cells during infection by a meningitic *C. sakazakii* strain. Previous paper *report the persistence of C. sakazakii in* in macrophages. Moreover, in comparison with *Escherichia coli* K1, the *C. sakazakii* invades brain endothelial cells more efficiently[9].

Our study results analysis will be helpful as first step of understanding the pathogenicity of infection of brain by *C. sakazakii* infection and further validation in the future. Overall, we evaluate the response to the *C. sakazakii* strain 767, using different infection time (60, 90 and 120 minutes). . According to these conditions, 2500 proteins were quantitated.

The most important finding of our study was that infection with the 767 *C. sakazakii* strain induced the most profound changes to the host proteome, providing important directions for studying the mechanism of infection, pathogenesis and host response in *C. sakazakii* meningitis.

57 proteins were differentially expressed out of 2500 proteins in response to the three time courses of exposure. From important 18 proteins, we selected four down regulated expression (gate keeping movement, cell protection, nucleoside triphosphate synthesis and oxygen transport). Meanwhile the up regulated selected proteins play are role in : promotion of autophagy and inflammation, natural killer T cell mediation, , regulation during differentiation and peptidase activity (Table 1). These molecular changes suggestion an abnormal processes during infection of meninges by *C. sakazakii strain.*

One of the most important upregulated protein expressed with high fold change during the serial times of exposure to *C. sakazakii* was Brain acid soluble protein 1 (BASP1), which is known to play a vital role during differentiation of cells.It has been revealed that BASP1 up-regulation occurs during differentiation of podocyte cells of adult kidney [10]. Complimentary methods *in vivo* and *in vitro* have confirmed that co- expression of BASP1, growth associated protein 43 and drebrin mediates caspase-3 cleavage, is necessary to activate apoptotic cells[11]. Interestingly, this protein is also predicted to be involved in the cell junction component process. It is commonly known that tight junction disorder induces cell damage, particularly in brain cells[12].

These observations suggest a similar mechanism happening in the HBMEC response when exposed to the meningitic *C. Sakazakii* strain. At the gene level, the tight junction pathway was predicted to be activated in HBMEC cells at 120 minutes post-infection with strain 767. This finding is in agreement with study that reported that *C. sakazakii* entry is enhanced upon disruption of the tight junctions, which is an important feature of systemic infection in neonates and infants[13].

Another upregulated protein that is assumed to play a critical role in cell apoptosis capacities is Annexin A11. The Annexin had been used as reliable marker to detect apoptosis resulting in bacterial meningitis in several studies[14,15,16]. According to gene ontology, the apoptosis pathway was predicted to be activated by several genes after 120 minutes post-infection. Previous studies have demonstrated that *C. sakazakii* infection upregulated several genes related to apoptosis[1].

The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) protein was observed in several pathways (Fig 3). Studies have shown that bacteria develops different strategies to inhibit apoptosis, including NFKB upregulation, inhibition of caspase 3 activation and prevention of cytochrome c release[17]. Remarkably, apoptosis inhibition is an important mechanism of pathogenicity that enables the bacteria to replicate inside host cells[18]. Further studies need to be undertaken to understand the role of NFKB at the gene level, which may be useful in detecting whether *C. sakazakii* postpones the apoptosis process. Based on protein ontology, it was found that most cellular components, biological processes and molecular functions were predicted to upregulate in HBMEC cells after *C. sakazakii* infection. Intercellular communication which is an essential hallmark of multicellular organisms can be mediated through direct cell–cell contact or transfer of secreted molecules.

This communication involves intercellular transfer of extracellular vesicles. Notably, more than 50% proteins expressed differently in HBMEC cells at 120 minutes post-infection were predicted to activate the extracellular vesicle exosome process (Fig 2B). This is similar to what happens in brain tumours, which can use extracellular vesicles to secrete factors, allowing them to escape from immune surveillance [19]. *C. sakazakii* probably has a similar role to extracellular vesicles in brain cell functions, creating a good chance to colonise the cell. Such pathway activation in response to meningitic strain 767 would explain the ability of this strain to survive within human macrophages and microglial cells.

Another cellular component predicted to be upregulated in HBMEC cells, resulting in *C. sakazakii* infection, is the cytoskeleton. There are five proteins involved in this cellular structure that expressed differently in infected compared to uninfected HBMEC cells. It is known that the cytoskeleton comprises three types of fibers, namely microfilaments, microtubules and intermediate filaments. The results of several studies clearly demonstrated that: to invade host cells, the *Cronobacter* uses microfilaments mostly Actin microfilaments and microtubules. Moreover, Under actin-depolymerising agent cytochalasin D exposure *C. sakazakii* invasion was totally inhibited[13]..

Different signal transduction processes are incriminated *in C. sakazakii* invasion of HBMECs. It involves the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K/Akt) signalling pathway also plays a vital role to initiate the rearrangement of actin filaments and subsequent bacterial internalisation[1].

Brain cell death might occur through a combination of bacterial activity and host response. The synergistic effects is possible, since the meningitic strain has the ability of invasion/attachment, translocation, cytotoxicity, IL-8 stimulation and resistance and survival inside the macrophage cells are equivalent; these factors increase the risk of pathogenicity of this bacterium even in healthy conditions. Our study highlights that the *C. sakazakii* virulence factors have a large protein panel and diverse pathogenic expression. These proteins are involved in host defences resistance, colonisation and adherence, invasion and tissue damage.

To sum up, our study data provide some important leads to study the mechanism of infection, pathogenesis and host response in *C. sakazakii*. The interactions between bacterial virulence factors and host response systems that initiate *C. sakazakii* meningitis would aid in our understanding of its pathogenesis. Therefore, we concluded that *C. sakazakii* caused meningitis through the three possible mechanisms, as follows: first , *C. sakazakii* may directly affect the expression of important proteins, such as BASI and Annexin. Second, it may promote brain health by increasing brain defenses (immune and/or innate); and third, a simultaneous increase in *C. sakazakii* might upregulate several genes related to apoptosis, tight junction production, and various signalling pathways.

Overall, our result may help to better understanding *C. sakazakii* pathogenicity, which can cause irreparable damage to a new-born baby's brain. Further studies are needed is warranted to establish the link of each of these proteins with the pathogenicity of *Cronobacter*. Subsequently, it could help to better control this pathogen by developing molecular targeted therapy.

CONCLUSIONS

The data of our study highlights important concepts about the mechanism of infection and host response in C. sakazakii. A number of proteins that are differentially expressed in HBMEC cells in response to meningitis isolate were identified in our study. Elevated levels of proteins

associated with the tight junction and apoptosis pathways were found and an increase in these proteins could be a reflection of enhanced cytokines production, and thus, enhanced inflammatory processing. Furthermore, the elevation in extracellular vesicular exosome proteins that was observed may prevent the intrusion of the bacterium into the system *via* the adhesion and aggregation of pathogens. This study concluded that many cell death processes response to the *C. sakazakii* meningitic strain, but were still not enough to kill the brain cells.In summary results of our study may facilitate a better understanding of this bacterium, which can cause irreparable damage to a new-born baby's brain.

Conflict of interest: Nil

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