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Food Safety Risks and Contributing Factors of *Cronobacter* spp.

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ABSTRACT

Cronobacter species are a group of Gram-negative opportunistic pathogens, which cause meningitis, septicemia, and necrotizing enterocolitis in neonates and infants, with neurological sequelae in severe cases. Interest in *Cronobacter* has increased significantly in recent years due to its high virulence in children. In this review, we summarize the current understanding of the prevalence of *Cronobacter* species in several important food types. We discuss the response mechanisms enabling persistence in adverse growth conditions, as well as its pathogenicity. We emphasize the food safety concerns caused by *Cronobacter* and subsequent control methods and clinical treatments.

1. Introduction

The *Cronobacter* genera (formerly known as *Enterobacter sakazakii* (*E. sakazaki*)) are Gram-negative non-spore forming bacilli belonging to the family Enterobacteriaceae. They colonize both animal and human intestines [1]. Iversen et al. [2] proposed the reclassification of *E. sakazakii* into the genus *Cronobacter* following biochemical and DNA hybridization analyses in 2008. At present, *Cronobacter* comprises seven species. *C. sakazakii* and *C. malonaticus* are the two species reported to cause serious illnesses. *C. muytjensii*, *C. dublinensis*, *C. turicensis* and *C. universalis* are of less clinical importance, and *C. condimenti*, has rarely been reported.

Considerable attention has been given to *Cronobacter* infections of neonates. Beginning in 1961, *Cronobacter* was first reported in hospitalized infants in the United Kingdom. In 1979, *Cronobacter* bacteremia cases were reported among newborns in Macon, USA. Further *Cronobacter* infections have been reported in the Netherlands (1983, 1987), Greece (1987), Iceland (1989), and the United States (1989 and 2001). In 2001, a cluster of *Cronobacter* infections enabled the US Center for Disease Control and Prevention (CDC) to undertake a traceback investigation which identified contaminated powdered infant formula (PIF) as a major source leading to infant infections [3].

Despite the attention given to infant cases, *Cronobacter* can infect individuals of all age groups. *Cronobacter* infections in adults result in mild gastrointestinal symptoms, diarrhea, and urinary tract infections. The elderly [4] and immunocompromised adults are the most susceptible to *Cronobacter*.

Newborn infants within two months of birth, especially underweight premature infants [5], have a less acidic stomach than adults and an inadequate immune response. Subsequently, their risk of bacterial infections is higher, with case fatality rates ranging from 50% to 80% [6]. In 2002, the International Commission on Microbiological Specification for Foods (ICMSF) [7] defined *Cronobacter* as "severe risk for a restricted population, representing a threat of death or chronic sequels of long duration." The US CDC estimated that four to six babies are infected by *Cronobacter* each year in the United States. This is an under-estimate since there is no requirement to report *Cronobacter* infections to governmental health departments.

In many countries, infantile *Cronobacter* infections have been frequently traced to the consumption of PIF contaminated with *Cronobacter*. However, there has been no definitive clinical report of *Cronobacter* being linked to a related PIF brand in China as few molecular source tracking analyses have been conducted. This outcome may be because the tracing of clinical infections due to

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foodborne *Cronobacter* in China is under-performing and the monitoring system is still improving [8].

Cronobacter infections involve adherence to host cell surfaces, followed by invasion of the intestinal and blood–brain barriers (BBB; Fig. 1 [9–11]). Clinical symptoms include fever and lethargy [12], and severe infections may result in conjunctivitis, bilious septicaemia, urosepsis, or meningitis [13]. Although patients infected by *Cronobacter* can recover after antibiotic treatment, they often suffer severe neurological sequelae and developmental disabilities.

Therefore, in order to fully understand the potential risk and pathophysiology of *Cronobacter*, this paper reviews the prevalence and distribution of this pathogen in foods, its persistence in adverse environments, the expression of virulence factors which contribute to its food safety concerns, and the need for improved clinical management.

2. Food contamination

Cronobacter is widely distributed in nature, occurring in water, soil, animal, and human feces [14]. Researchers are exploring ecological niches for *Cronobacter* and its transmission routes, epidemiology, and pathogenic mechanisms. Consequently, determining the prevalence of *Cronobacter* in food will support microbial source tracking studies and should reduce the risk of further *Cronobacter* outbreaks.

2.1. Infant foods

Since the first case of neonatal infections caused by *Cronobacter* in the United Kingdom [15], there have been many reported outbreaks of *Cronobacter* infections linked to the infant foods worldwide [16,17]. Therefore, monitoring the presence of *Cronobacter* in PIF and other infants foods is important. Liu et al. [18] found *Cronobacter* in 87 (12.6%) PIF from Fuyang, Anhui Province in China. These products failed to meet good manufacturing practices and quality assurance schemes. In 2018, the Rapid Alert System for Food and Feed (RASFF) of the European Union reported the contamination of multiple batches of PIF from various brands. It is thought that the contamination originated from the use of contaminated raw materials which had been exported to PIF manufacturers in countries such as China and France. PIF is not the only infant food of concern, as the contamination rate of *C. sakazakii* in the infant supplementary foods (ranges from 1.2% to 27%) is similar to that of PIF (ranges from 0.9% to 23.1%). These supplementary foods include formula rice flour, nutritional biscuits, and flour products for infants [8].

Since cases of neonatal infection have been linked to reconstituted contaminated PIF [16], *Cronobacter* was classified as Class A pathogen by the Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) in 2004 [19].

Contamination of PIF can occur through the addition of non-sterile raw materials to formula powder or ingredients after pasteurization. The manufacture of PIF production may entail dry-blending and wet-blending of ingredients before spray drying. The dry-blend process usually does not use high temperature treatment, subsequently low levels of a pathogen may persist in the final product. Such low level contamination can be missed in a random sampling inspection. In addition, aerosols generated by air inlets and outlets, and operators may generate exogenous *Cronobacter* in the spray drying tower during the wet-blend spray drying process [20]. Areas within a PIF production facility associated with higher *Cronobacter* contamination include the spray dryer, powder from the floor, fluidized beds, and air filtration facilities [21]. Consequently, pasteurization only inactivates the *Cronobacter* in earlier stages of PIF production, but is ineffective in controlling contamination from downstream ingredients, filters, spray drying tower and other processing equipment. *Cronobacter* does survive spray-drying [22,23]. Once the PIF production environment is contaminated with *Cronobacter*, it can persist for years [24].

2.2. Ready-to-eat fresh foods

In addition to infant foods, *Cronobacter* is also detected in foods of Japanese cuisine [25], vegetables, cheese, and various cooked meat products [26]. Plants are likely to the main ecological niche for *Cronobacter* [27]. This would explain the high prevalence in ready-to-eat vegetables (30.27% positive) [28], and salads (8.2%)

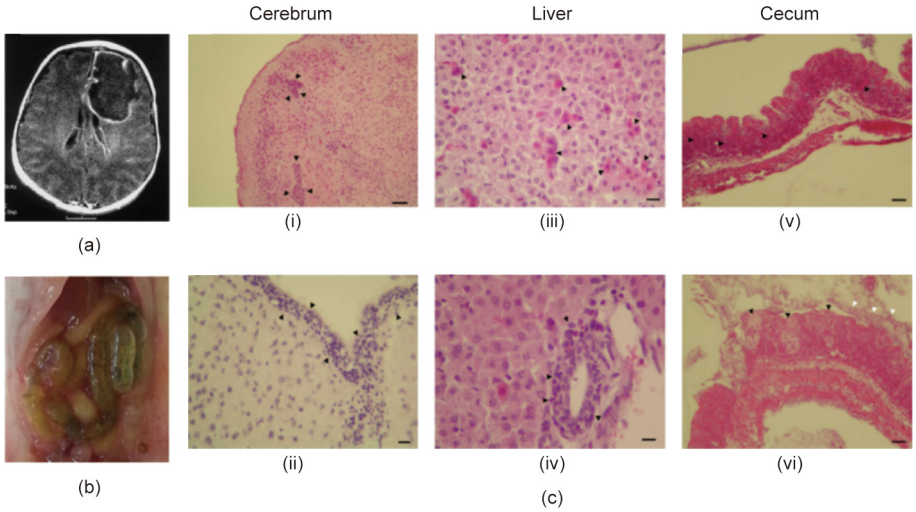

Fig. 1. (a) Axial postcontrast T1-weighted magnetic resonance image (MRI) of a *Cronobacter* brain infection with a large ring-enhancing fluid-containing lesion in the left frontal lobe in a neonate (reproduced from Ref. [9]). (b) Rat pups fed formula contaminated with *Cronobacter* developed neonatal necrotizing enterocolitis (NEC) (reproduced from Ref. [10]). (c) Histopathological findings of *Cronobacter* infected mice by hematoxylin-eosin staining: (i) gliosis (black arrows, scale bar = 100 μm) and (ii) meningitis (black arrows, scale bar = 50 μm) of cerebrum section; (iii) hepatocytic degeneration (black arrows, scale bar = 50 μm) and (iv) inflammation (black arrows, scale bar = 50 μm) of liver section; (v) inflammation (black arrows, scale bar = 100 μm); and (vi) degeneration (black arrows) and bacterial colonies (white arrows, scale bar = 100 μm) of cecum section (reproduced from Ref. [11]).

[29]. There is also a seasonality in their presence on these foods. The commercial seasons for salad vegetables, fresh juice, and fresh fruit are usually in spring and summer. These warmer months may provide favorable conditions for pathogen replication and persistence in these foods.

Good hygienic practices are required in food preparation as *Cronobacter* in raw ingredients, may cross contamination in ready-to-eat foods if proper cleaning procedures for cutting boards, knives, and utensils used for processing and holding cooked foods are not followed.

2.3. Dehydrated foods

Cronobacter are highly resistant to desiccation, and this may explain the persistence and higher number of infection due to PIF by this organism compared to other Enterobacteriaceae. A study of 1012 samples of dehydrated rice powder samples from 14 manufacturers in China, gave an average of detect rate of 7.5%, though the samples from one manufacturer was as high as 28.8% [30]. A five-year survey of *Cronobacter* contamination in foods produced or sold in the Netherlands, showed that the detection rate in dry cereal foods and spices were about 4% and 3%, respectively [31]. Furthermore, the detection rate of *Cronobacter* in Chinese spices was as high as 57% [32] and a high prevalence (6.3%) in seasoning mixtures [33]. Therefore, the addition of spices to foods without further cooking may pose a hazard to consumers especially those who are immuno-compromised.

Given the prevalence of *Cronobacter*, the food industry, especially manufacturers of infant foods, needs to control this pathogen. For example, raw materials and the processing environment must be effectively separated and regularly cleaned.

3. Environment stress

Foodborne pathogens are often exposed to various adverse environments between food production and the consumer's meal (Fig. 2). In response to drying, heat, and acid shock, *Cronobacter* synthesizes substance compounds such as trehalose, proline, glycine, betaine, glucose, *N*-acyl-homoserine lactones, pigments, and specific proteins [34] (Fig. 3). These stress responses have been further studied as they are important in the persistence of the pathogen in food production, and subsequent increased exposure to infants.

3.1. Desiccation resistance

Cronobacter are more tolerant to desiccation than *Escherichia coli (E. coli)*, *Salmonella*, *Listeria*, and other Enterobacteriaceae species [35]. The viable counts of *Cronobacter* in PIF declined approximately 2.4 log cycles after five months of storage at room temperature, and then declined by only 1.0 log cycle after the subsequent 19 months, and some strains even survived in PIF for as long as two years [36].

Breeuwer et al. [37] proposed that *Cronobacter* increased intracellular osmotic pressure through the accumulation of metal ions such as K^+, and synthesis of compatible soluble substances including trehalose, proline, and glycine, leading to maintaining water membrane and therefore reduce cell dehydration. *Cronobacter* OpuC and ProP system encoded by the *opuCABCD* operon and the *proP* gene transports proline, glycine, and betaine, and membrane-binding protein ProU system encoded by *proVWX* operon, which can transport osmoprotectants into the cell to resist desiccation, are up-regulated during desiccation response [38]. Moreover, the trehalose content of *Cronobacter* in stationary phase was 0.04 $\mu mol \cdot mg^{-1}$ under normal culture conditions, and increased more than five-fold to 0.23 $\mu mol \cdot mg^{-1}$ after desiccation. In contrast, no accumulation of trehalose was detected in other Enterobacteriaceae under these desiccation stress conditions. *C. sakazakii* ATCC 29544 desiccation resistance factors have been analyzed using sobaric tags for relative and absolute quantification (iTRAQ). This revealed the up-regulation of the trehalose synthesis gene operon *OstAB* and trehalose transporter genes *TreABCF* [38], whose products could protect phospholipid membrane and outer membrane proteins under desiccation conditions [39].

Physiological changes caused by hypertonic and desiccated environments are similar, and can lead to confusion between osmotic and desiccation responses. Stress response of *Cronobacter* under physical desiccation and 1 $mol \cdot L^{-1}$ NaCl hyperosmotic media observed by Riedel and Lehner [40] using two-dimensional gel electrophoresis (2-DE) showed that the expression of the periplasmic protein OsmY was up-regulated in hypertonic culture, while amino acid biosynthesis and their transporters such as γ-glutamyl transpeptidase were significantly down-regulated. This resulted in slowed growth and decreased metabolism. Expression of heat shock protein, DNA-binding protein Hns (heat stable nucleoid-structuring protein), chaperonin GroES, and OmpA are continuously up-regulated which helps cells to cope with desiccation. In addition, capsule-producing *Cronobacter* were recoverable from PIF after 2.5 years, unlike non-capsulated strains [36]. As the main component of *Cronobacter* biofilms, extracellular capsular polysaccharides enable cells to form biofilms on stainless steel and glass substrates, thus enhancing their ability to survive [41,42]. Our team [43] discovered that *C. malonaticus* biofilm could be positively affected by an oligogalacturonate lyase gene, which improved the adaptability of the strain under stressful osmotic conditions.

3.2. Heat resistance

Rehydrating PIF with boiled water and cooled to 70 °C is an important step to avoid consumption of PIF contaminated with *Cronobacter* [44]. The optimum growth temperature for *Cronobacter* ranges from 5.5 to 47.0 °C, and it can survive thermal conditions between 54 and 64 °C [45]. The decimal reduction time (*D*-value) of *Cronobacter* at 58 °C ranges from 0.39 to 0.60 min as reported by Breeuwer et al. [37], and a few *Cronobacter* strains isolated from infant foods remained viable at 65 °C for 5 min [46] suggesting that PIF should be reconstituted with water > 70 °C, rather than the water with mild heat.

Riedel and Lehner [40] and Asakura et al. [47] proposed that Mfla-1165 and translation initiation factor *infB*, which is specifically up-regulated in heat-resistant strains compared to heat-sensitive strains, could be used as a molecular marker for heat-resistance. Moreover, carriage of the *thrB–Q* genomic island could enhance the viability of *Cronobacter* at 58 °C, and differential expression of *rpoS* gene may be another reason for the differences in thermotolerance reported in *Cronobacter* strains [48]. Similarly, due to similar physiological regulatory responses, *Salmonella* exposed to a low-moisture environment can exhibit enhanced resistance to high temperature processes through cross-protection [49]. Desiccation resistance of *Cronobacter* also leads to increased heat tolerance [50,51]. Despite the importance of this topic with respect to the persistence of *Cronobacter* after rehydration of PIF with hot water, few studies have focused on the interaction between desiccation resistance and heat resistance.

3.3. Acid resistance

Generally, pH of gastric fluid in adults is lower than two, whereas that in infants fed PIF it is about 3.6. This difference in pH is significance as *C. sakazakii* is still able to grow after exposure to pH 3.0 for 90 min [52]. Consequently, *Cronobacter* in contaminated PIF or other supplementary foods can survive the gastric acid

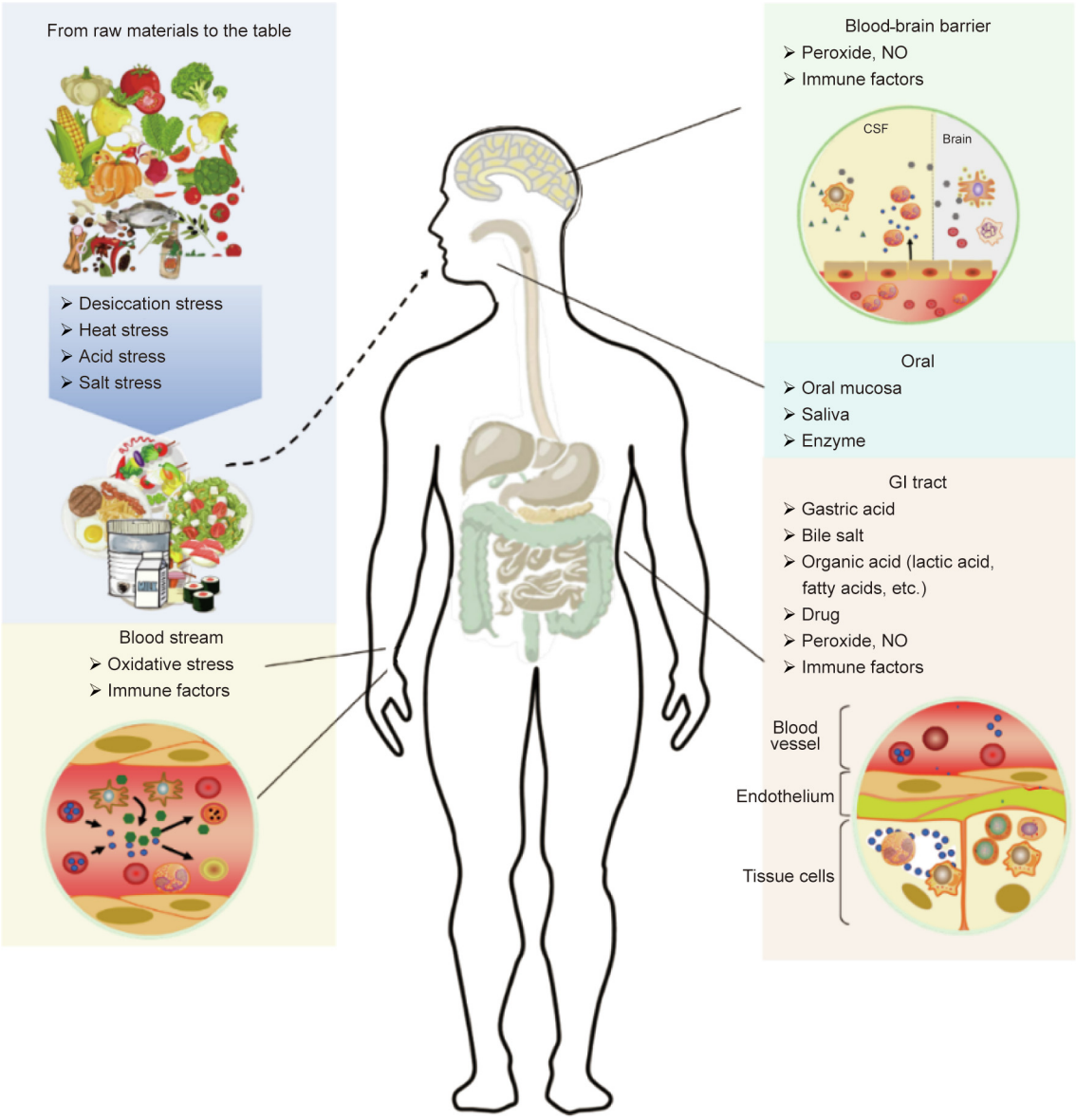

Fig. 2. Diverse microbial stresses from foods and the human host. Acid is a common food preservative and a disinfectant in the stomach; salt is a common food preservative and a natural host defense that exerts osmotic stress; low moisture activity from grain byproduct or powder foods and the food heating process create an unfavorable environment for the growth of foodborne pathogens; the oral mucosa is the first protective barrier which the bacteria are exposed to which occurs during food intake, and enzymes produced by the oral cavity and stomach then can also inhibit growth; organic acids, drugs, oxides, and immune factors in the gastric intestinal (GI) tract are the inevitable stresses faced by bacteria; oxides and nitric oxide (NO) are the substances released by immune cells; immune factors are the host defense responses induced by immune cells. CSF: cerebrospinal fluid.

barrier and enter the small intestine causing intestinal inflammation such as necrotizing enterocolitis.

Acid treatment delays the growth of *Cronobacter*, due to the accumulation of intracellular anions leading to damage to DNA and proteins. However, the normal bacterial morphology is restored demonstrating tolerance to acid stress and recovery from cellular injury [53]. *Cronobacter* has a series of molecular strategies to deal with acid stress. This includes the up-regulation in the expression of *cpxR*, *ompR* [54], *rpoS*, *hfq* [55], *grxB* [53], and *phoP*/*phoQ* [56]. These acid stress responses are involved in sensing and repairing damaged proteins and nucleic acids, and by regulating oxidative stress.

Bacteria have a common stress response mechanism which leads to cross-resistance effects in their tolerance to various environmental stresses. This makes their stress-resistance regulatory pathways complex and diverse. Therefore, future research on the stress responses of *Cronobacter* should not only focus on each independent regulatory and metabolic pathway of related proteins and genes associated with a specific environmental stress response, but also on centralized stress regulatory systems and cross-protection mechanisms.

3.4. Antimicrobial resistance

Cronobacter isolated from multiple food sources have been screened for antimicrobial sensitivity. Resistance has been found to many clinically important antibiotics, including penicillin, clindamycin, cephalosporin, vancomycin, and tetracycline [57]. Furthermore, an increasing number of multidrug-resistant *Cronobacter* have been reported with inducible AmpC

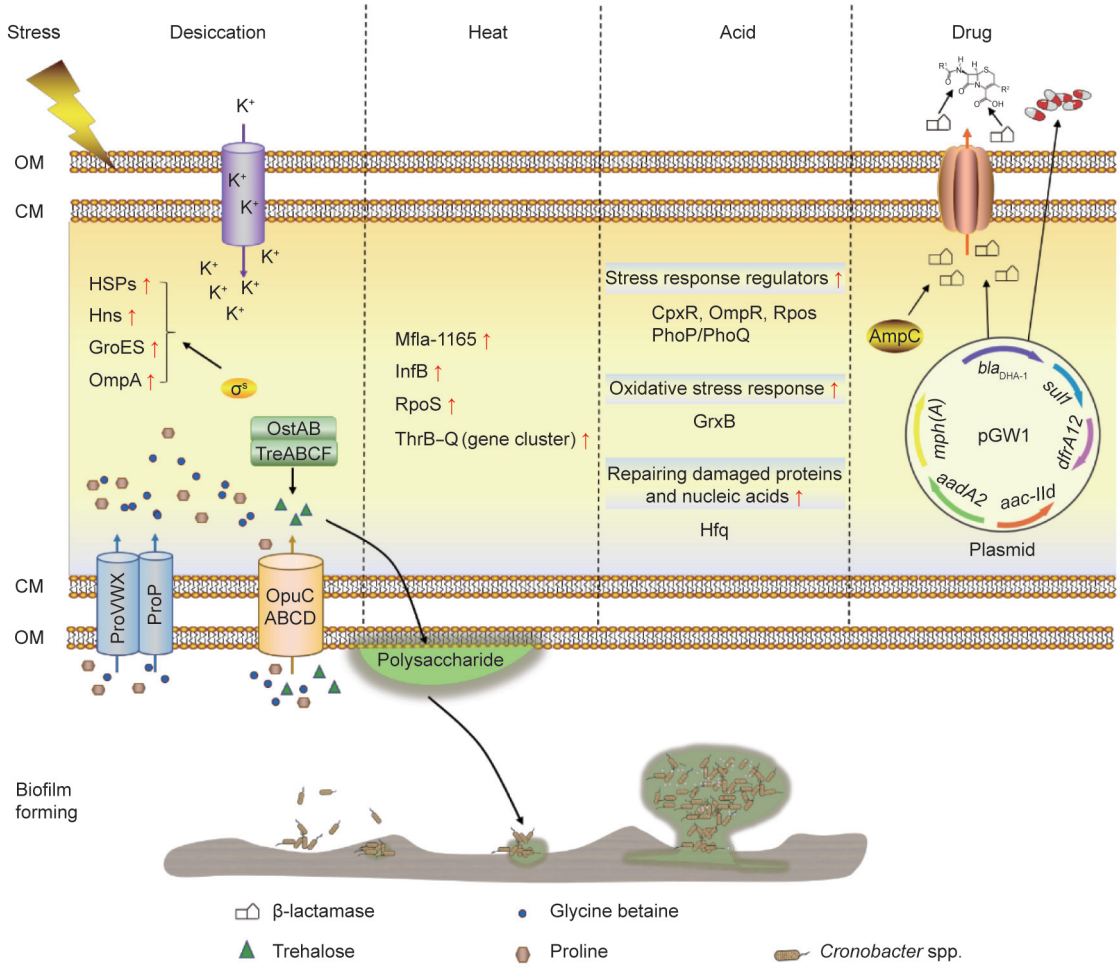

Fig. 3. Response of *Cronobacter* spp. under adverse environment. OM and CM represent outer and cytoplasmic membrane, respectively; HSPs: heat shock proteins; Hns: heat-stable nucleoid-structuring protein.

β-lactamases and the horizontal transmission of drug-resistance genes.

Caubilla-Barron et al. [58] identified three *Cronobacter* strains with β-lactamase activity isolated during a neonatal intensive care unit (NICU) outbreak. Strains isolated from the tracheal secretion and blood of deceased individuals were resistant to ampicillin, cefuroxime sodium, cefotaxime, and amoxicillin/clavulanic acid. Meanwhile, the isolate from a neonatal meningitis case showed the presence of bla_{DHA-1} encoding AmpC β-lactamase which was carried on a plasmid. This conferred cephalosporin resistance. Also five new drug resistance genes (*mph*(*A*), *sul1*, *aadA2*, *dfrA12*, and *aac-IId*) were detected [59].

Colistin has been used as a growth promotant in livestock feed in some countries such as China for many years. Recently, *mcr-1* and *mcr-10* were identified as plasmid-borne colistin resistance genes in *Cronobacter* [60,61]. The spread of plasmid-encoded antimicrobial resistance and the acceleration of the rate of antimicrobial resistance may be attributed to the overuse of antibiotics [62–64]. Such antimicrobial resistance is an increasingly serious threat to global public health. In order to reduce the disturbance of normal intestinal flora and the spread of *Cronobacter* antimicrobial resistance, researchers have investigated the use of novel or combinational therapy. For example, metabolites produced by *Bifidobacterium* has been shown to relieve intestinal inflammation caused by *C. sakazakii* in mice [65], and *Lactobacillus rhamnosus* can inhibit the growth of *C. sakazakii* through altering cell membrane permeability [66]. Additionally, sub-inhibitory concentrations of citral could increase the sensitivity of *C. sakazakii* to ampicillin and cefoxitin [67]. Lipoic acid [68] is able to inhibit *C. sakazakii in vitro,* showing that probiotics and their products could be further developed as strategies for the effective treatment of *Cronobacter* infections.

Cronobacter specifically acquire K^+, trehalose, proline, and glycine from extracellular matrix, and simultaneously enhance the synthesis of trehalose, proline, and glycine *in vivo* during desiccation. Moreover, heat shock protein HSPs, DNA-binding protein Hns, chaperonin GroES, and outer membrane protein OmpA are also beneficial to desiccation tolerance; Mfla-1165, translation initiation factor *infB*, sigma factor *rpoS*, and *thrB–Q* genomic island are involved in thermotolerance; *Cronobacter* withstand against acid stress through increasing the expression of stress response regulators, chaperone, genes related to oxidative stress response; secretion of β-lactamase and horizontal transfer of drug-resistance genes mediated by pGW1 plasmid are essential for the viability of *Cronobacter* under drug treatment; trehalose is an important component of exopolysaccharide in biofilm, which make outstanding contribution in *Cronobacter* survival under diverse adversities.

4. Pathophysiology

Gastrointestinal pathogens usually cause disease by crossing a number of physical, chemical, microbial, and immune barriers of

the host. *Cronobacter* encodes many virulence factors involved in the pathogenic process, facilitating adhesion, invasion and disruption to intestinal barrier and BBB (Fig. 4). Researchers believed that the high infection rate of *Cronobacter* in newborns resulted from the immature intestinal barrier, which makes the intestinal mucosa vulnerable to *Cronobacter* leading to bacterial translocation into other tissues [69]. Accompanying with intestinal immune dysfunction, *Cronobacter* triggers a series of cytokines to cause an inflammatory cascade reaction, which causes further intestinal injury and eventually intestinal necrosis. In addition, *Cronobacter* can enter the bloodstream (bacteremia), invade microvascular endothelial cells of the brain, and cross the BBB to cause life-threatening meningitis [69].

4.1. Attachment

Attachment of *Cronobacter* to host tissue receptors is the initial step in disease, such as using fimbriae to adhere to the surface of intestinal epithelial cells [70], followed by host cell invasion [71]. The fimbriae are encoded by the *sfp* gene cluster [72], and P-fimbriae increase the infection rate of neonatal meningitis [73]. In addition, putative proteins ESA_00281 and ESA_00282 in *C. sakazakii* ES5 strain are associated with the adhesion process, and flagellin proteins FlhA, FliG and FliC involved in self-agglutination [74]. Having crossed the intestinal barrier, the bacteria invade the circulatory system, causing severe systemic infection [75] and may penetrate the BBB. Sub-inhibitory concentrations of lipoic acid inhibit bacterial motility swimming and swarming at the initial stage of *C. sakazakii* infection, thus reducing the invasion ability of the pathogen to host cells [68].

4.2. Invasion

The host immune balance, is perturbed by invasive pathogens. Often the host response is to suppresses abnormal cellular apoptosis by phagocytosis, the clearance of immune cells, secretion of immune factors, cell mucin, and simultaneously enhance the expression of tight junction protein to minimize damage [76]. However, *Cronobacter* is able to invade intestinal epithelial cells, entering the blood, and subsequently infect the liver and spleen, and cross the BBB into the brain [77].

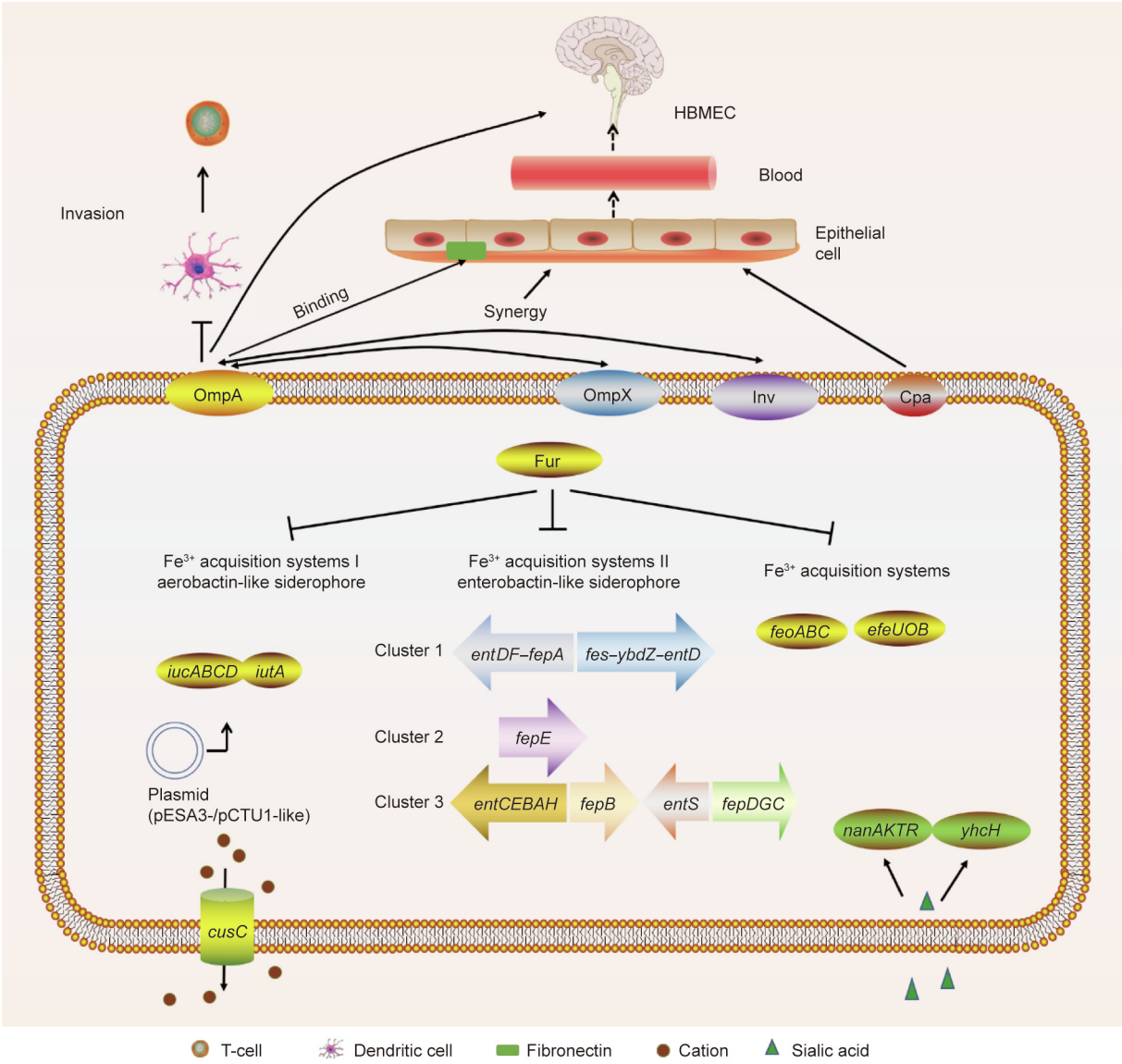

Fig. 4. Virulence factors involved in *Cronobacter* spp. invasion of host cells. HBMEC: human brain microvascular endothelial cells.

Outer membrane protein, lipopolysaccharide, enterotoxin, invasion, and plasminogen activator produced by *Cronobacter* are the main factors for invasion to host. In order to aggravate infection, *Cronobacter* survive and multiply in host through iron acquisition systems, cation efflux system, quorum sensing systems, and catalyzing and utilizing sialic acid.

4.2.1. Outer membrane protein

It is general accepted that cellular invasion by *Cronobacter* is closely related to expression of outer membrane proteins (OMPs). These proteins are embedded in the outer phospholipid bilayer of Gram-negative bacteria. The outer membrane protein A (OmpA) of *Cronobacter* is the receptor for phages and F-fimbriae mediated conjugation. Mittal et al. [78] found that OmpA contributed to neutrophil infiltration, hemorrhage, and gliosis. This indicated that OmpA enables *Cronobacter* to proliferate in the blood and traverse the small intestinal barrier of a newborn rat. OmpA binds to fibronectin leading to invasion of the brain microvascular endothelial cells, and transfer through the BBB to induce cell apoptosis and meningitis [79]. Furthermore, invasin mediates *C. sakazakii* invasion of visceral organs of the host. There is a synergistic effect with the pathogenicity of OmpA, *in vitro* and *in vivo* [80].

Outer membrane protein X (OmpX) is a protein with small molecular weight, which cooperates with OmpA in the invasion by *C. sakazakii* of intestinal tissue. The absence of OmpX results in a reduction of *C. sakazakii* invasion and adhesion to Caco-2 and INT-407 intestinal cell lines [81]. Microarray analysis has shown that *Cronobacter* secretes outer membrane vesicles (OMVs) enveloping these OMPs, which are taken up by intestinal cells, increasing cell proliferation and stimulating the host's innate proinflammatory response [82].

4.2.2. Lipopolysaccharide

Lipopolysaccharide (LPS), also known as O-antigen or endotoxin, is embedded in the outer membrane of the Gram-negative cell wall. It is released when bacteria die or subject to physicochemical attacks. Previous studies have shown that LPS exposure can stimulate the immune response of the host and improve immunity, and that a high concentration of LPS or long-term exposure to low concentration of LPS, will disorganize a host's homeostasis and induce inflammation such as diabetes and fatty liver. Ingested LPS reduces the permeability of the infant intestinal wall leading to bacterial invasion and systemic infection [69]. LPS is an important virulence factor of *Cronobacter*, which after being recognized by toll-like receptors TLR2 or TLR4 triggers a series of cascade reactions and the expression of pro-inflammatory factors [69]. Our group found that intraperitoneal injection of *C. sakazakii* LPS resulted in the disorder of gut flora, and the up-regulated expression of intestinal tight junction protein (Claudin-4 and Occludin), TLR4, and inducible nitric oxide synthetase iNOS, and damage to intestinal tissue in mice (the data was unpublished). Notably, bacterial LPS is difficult to eliminate completely on account of its heat-resistant during the PIF processing leading to the presence of endotoxin in the end-product [69]. However, safety standards for the detection or limitation of LPS in PIF have not been implemented. Therefore, infants with immature innate immune systems may be at increased risk of bacteremia by the intestinal flora or endotoxemia if they ingested milk powder containing high levels of LPS.

4.2.3. Enterotoxin

Pagotto et al. [83] first described that four out of 18 *Cronobacter* isolates produced enterotoxin-like compounds, which along with LPS caused inflammation. These enterotoxin-like compounds increased cyclic adenosine monophosphate (cAMP) level in intestinal epithelial cells, leading to water, sodium, chlorine, and potassium bicarbonate in intestinal cells to be continuously secreted into the lumen causing diarrhea. Raghav and Aggarwal [84] identified an enterotoxin-like compound with a molecular weight of 66 kDa, and proposed that it may be a risk factor in PIF contaminated by *Cronobacter* as it was still active after being treated at 90 °C for 30 min. Studies have shown that the survival rate of challenged mice was only 33% after 72 h when 10^4–10^5 colony forming units (CFU) per milliliter of *Cronobacter* were injected into mice, while all mice died when the injection reached 10^7 CFU·mL^{-1} cells [85]. Nevertheless, the genes encoding the toxin are still unknown, further research focused on the properties and formation mechanism of *Cronobacter* enterotoxin are needed.

4.2.4. Iron acquisition systems

As an important coenzyme factor in *Cronobacter*, iron participates in many metabolic reactions such as electron transfer and cell respiration. Under iron-limiting conditions, *Cronobacter* secretes siderophores with a high affinity for Fe^{3+}. The Fe^{3+}-siderophore complex is transferred to the cytoplasm where the iron is released. Grim et al. [86] compared the iron acquisition systems in the genomes of 231 *Cronobacter* originating from various sources, and found that all *Cronobacter* isolates had genes encoding for aerobactin and enterobactin-like siderophores. The aerobactin-like siderophore gene clusters comprised *iucABCD*, and the receptor gene *iutA* are plasmid borne. In contrast, the enterobactin-like siderophore genes, namely *entD*, *fepA*, *fes*, *ybdZ*, *entF*, and *entCEBAH*, *fepB*, *entS*, *fepDGC*, and *fepE*, are at three different locations on the chromosome. In addition, *Cronobacter* species also express two ferrous transport systems Feo and Efe, encoded by *feoABC* and *efeUOB,* respectively. These are used to obtain Fe^{2+} from the environment. Expression of these iron transport systems is generally repressed via interaction of ferric uptake regulator (Fur) proteins binding to relevant promoter sites. The Fur family is a family of bacterial proteins involved in regulating metal ion uptake and in metal cell homeostasis. Fur proteins are responsible for controlling the intracellular concentration of iron in many bacteria. Even though iron is essential, its concentration must be carefully regulated over a wide range of environmental conditions. This is because high concentrations can be toxic due to the formation of reactive oxygen species.

4.2.5. Other virulence factors

With the development of proteomics and transcriptomics, various virulence factors of *Cronobacter* have been proposed. These are summarized in Table 1. Studies have shown that only *C. sakazakii* and *C. turicensis* contain genes *nanAKTR* and *yhcH* that encode for sialic acid utilization [87]. Therefore, the exogenous sialic acid presented in breast milk and PIF, as well as a component of brain gangliosides and intestinal mucus can be used as a carbon source for growth by these species. This may contribute towards the severity of neonatal meningitis and subsequent brain damage. Interestingly, the neonatal meningitic *E. coil* K1 pathovar uses sialic acid form capsules on the cell surface and protects the organism from the host immune response, but this mechanism has not been found in *C. sakazakii* [88]. In addition, *Cronobacter* producing capsule can produce copious amounts of extracellular polysaccharide composed of colonic acid (CA) and K-antigen. The K2:CA2 type capsule might be one of important factors leading to meningitis in newborns infected with *C. sakazakii* clonal complex 4 [88]. However, there are no experimental evidence supporting the relationship between capsule composition and meningitis. Whether the K2:CA2 capsule participates in transiting the BBB is still unknown. Therefore, preventing and controlling infections due to *Cronobacter* species is an important issue, requiring an improved understanding of *Cronobacter* pathogenesis.

Cronobacter produce plasminogen activator factor which provides resistance against the bactericidal activity of serum [89].

Table 1
Proposed virulence factors of *Cronobacter* species.

Virulence factor	Related genes	Functions
OMPs	*ompA, ompX*	Promoting adherence and invasion to host cells
Fimbriae	*sfp*	Promoting adherence to host cells
Flagellum	*flhA, fliG, fliC*	Participating in the automatic aggregation of pathogenic bacteria and chemotaxis
Sialic acid utilization	*nanAKRT, yhcH*	Contributing to colonization of intestinal tract by pathogenic bacteria and digestion of gangliosides
Efflux system	*cusC*	Encoding cation efflux operon, mediating invasion of brain microvascular endothelial cells
Iron acquisition systems	*Fur, iucABCD/iutA, entD, fepA, fes, ybdZ, entF, entCEBAH, fepB, entS, fepDGC, fepE, feoABC, efeUOB, eitCBAD*	Encoding iron acquisition systems and participating in pathogenic process together with plasminogen activator, *iucABCD/iutA* and *eitCBAD* are on a RepFIB plasmid
Plasminogen activator	*cpa*	Related to the resistance of serum and the spread and invasion of pathogenic bacteria
Diffusible signal factor (DSF) quorum-sensing	*rpfF*	Regulating cyclic diaguanylate (c-di-GMP) in cells and increasing the lethal rate

The diffusible signal factor (DSF) quorum sensing system mediated by *RpfF* can affect biofilm formation, colony morphology, and motility via regulating concentration of intracellular cyclic diguanylate (c-di-GMP). This also has an important role in the process of adherence and invasion by the bacteria [90].

4.3. Apoptosis induction

Hunter et al. [91] proposed that *Cronobacter* may induce apoptosis by regulating intracellular signaling pathways in mouse intestinal epithelial cells. The nitric oxide (NO) synthesis system is activated in IEC-6 intestinal epithelial cells to release a large amount of NO and cause apoptosis during *Cronobacter* infection (Fig. 5). Furthermore, increased apoptosis of intestinal mucosal epithelial cells induced by *Cronobacter* could cause the destruction of the intestinal barrier, leading to the permeability of intestinal barrier and consequent bacterial translocation. However, pretreatment of *Cronobacter*-infected mouse model with *Lactobacillus bulgaricus* can reduce the synthesis of NO and prevent the apoptosis of IEC-6 cells. Similarly, a decline in iNOS and Caspase-3 enzyme activity can be detected by citral pretreatment of Caco-2 cells prior to a *Cronobacter* challenge. This alleviated the production of NO and the apoptosis of intestinal epithelial cells to resist against inflammation caused by *C. sakazakii* [67].

4.4. Immune evasion

C. sakazakii can persist for 96 h in human U937 macrophages and even replicate inside them [10], which could lead to systemic infection [77]. Townsend et al. [92] injected *Cronobacter* into the brain of neonatal mice, indicating that infiltrating macrophages and neutrophils formed inflammatory aggregation to phagocytize bacteria, and that most *Cronobacter* strains persisted in macrophage cells for 48 h. *C. sakazakii sod* gene encoding superoxide dismutase SOD, whose activity also affects the pH value, neutralizes the reactive oxygen species (ROS) in macrophages. This reduces the ROS stress, and the host immune response, thus improving the survival of *Cronobacter* [93]. Furthermore, studies have shown that *Cronobacter* OmpA interferes with the maturation of dendritic cells. These dendritic cells induced less or even no T cell proliferation enabling *Cronobacter* to escape the host immune defense mechanism [94].

The deleterious effects of *Cronobacter* infection are caused through five crucial processes. First, *Cronobacter* induce the decreased expression of tight junction proteins (occludin and zona occluden (ZO)-1); second, *Cronobacter* induce pyroptosis through NOD-like receptor family pyrin-domain-containing protein 3 (NLRP3)/caspase1/apoptosis associated specklike protein containing a C-terminal caspase recruitment domain (ASC) signaling; third, *Cronobacter* induce apoptosis through caspase3 signaling; fourth, *Cronobacter* activate inflammasomes; fifth, *Cronobacter* release a large amount of NO to damage cells.

5. Prospect

Cronobacter is pathogenic across all age groups and can cause serious infections with a high mortality rate. After being recognized as category "A" pathogen in PIF due to the related food safety incidents, *Cronobacter* has attracted considerable attention from WHO, governments, and infant food manufacturers. Therefore, knowing the exogenous transmission features of *Cronobacter* in food, and tracing the organism back to contamination sources, will lead to improved food safety standards for the organism. Consequently, these strategies will reduce the risk of infections and outbreaks by *Cronobacter*.

Meanwhile, the pathogenesis of *Cronobacter* still needs further elucidation. First, the environmental stress pathways need to be better understood especially as processing may induce survival mechanism. Secondly, the K2:CA2 type capsule has been proposed as unique to strains causing neonatal meningitis infection. However, whether this is a causal relationship is unknown. Finally, the application of bacteriostatic substances such as aromatic essential oils, polyphenols, and materials originated from probiotics may also be a new strategy to address the issues of *Cronobacter* multidrug-resistant strains.

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Compliance with ethics guidelines

Na Ling, Xiuting Jiang, Stephen Forsythe, Danfeng Zhang, Yizhong Shen, Yu Ding, Juan Wang, Jumei Zhang, Qingping Wu, and Yingwang Ye declare that they have no conflict of interest or financial conflicts to disclose.

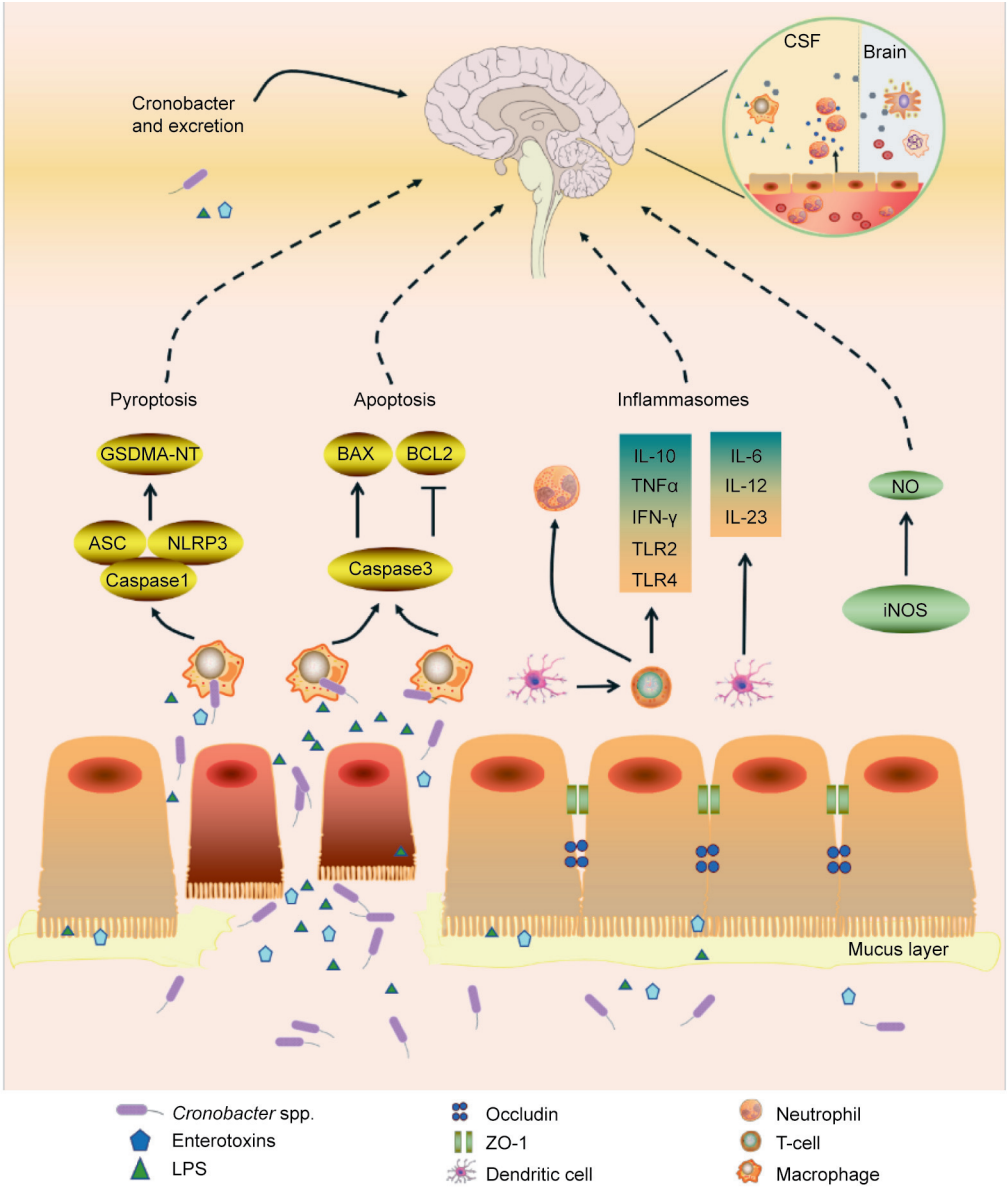

Fig. 5. Host cell barrier and immune response caused by *Cronobacter* spp. GSDMA: gasdermin A; NT: N-terminal; ASC: apoptosis associated speck-like protein containing a C-terminal caspase recruitment domain; NLRP3: NOD-like receptor family pyrin-domain-containing protein 3; BCL: B-cell lymphoma; BAX: BCL2-associated X protein; IL: interleukin; ZO: Zona occludens; TNF: tumor necrosis factor; IFN: interferon.

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