Insights into the mechanisms of *Cronobacter sakazakii* virulence

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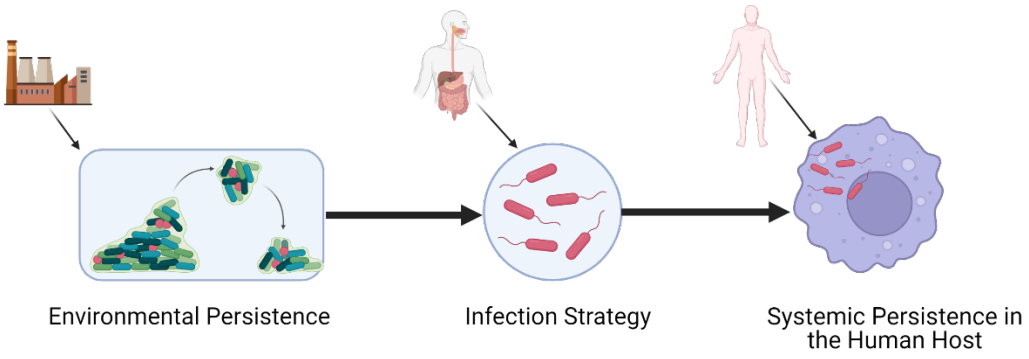
Abstract

Cronobacter species have adapted to survive harsh conditions, particularly in the food manufacture environment, and can cause life-threatening infections in susceptible hosts. These opportunistic pathogens employ a multitude of mechanisms to aid their virulence throughout three key stages: environmental persistence, infection strategy, and systemic persistence in the human host. Environmental persistence is aided by the formation of biofilms, development of subpopulations, and high tolerance to environmental stressors. Successful infection in the human host involves several mechanisms such as protein secretion, motility, quorum sensing, colonization, and translocation. Survival inside the host is achieved *via* competitive acquisition and utilization of minerals and metabolites respectively, coupled with host immune system evasion and antimicrobial resistance (AMR) mechanisms. Across the globe, *Cronobacter sakazakii* is associated with often fatal systemic infections in populations including neonates, infants, the elderly and the immunocompromised. By providing insight into the mechanisms of virulence utilized by this pathogen across these three stages, this review identifies current gaps in the literature. Further research into these virulence mechanisms is required to inform novel mitigation measures to improve global food safety with regards to this food-borne pathogen.

Key words: *Cronobacter sakazakii*, Virulence mechanisms, Pathogenesis, Environmental persistence, Systemic infection



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1. Introduction

Cronobacter species are Gram-negative, xerotolerant bacteria described as opportunistic pathogens predominately affecting those with compromised immune systems [1]. *Cronobacter* is primarily reported in neonatal infections, with an infant fatality rate of between 40-80% [2], while 20% of survivors develop neurological disorders [3]. Previously known as the single species *Enterobacter sakazakii*, *Cronobacter* genus has undergone several changes in nomenclature due to the advancement of phylogenetic analysis, including sequencing techniques. Of the seven known species, *C. sakazakii, C. malonaticus* and *C. turicensis* are reported to cause severe infection [4].

Open containers of contaminated powdered infant formula (PIF) are the most commonly identified source of *Cronobacter*-associated infantile infections, however contaminated expressed breast milk and neonatal feeding tubes have also been identified as reservoirs [5]. Regarding the production of PIF, *Cronobacter* isolates have been retrieved from both inert and bioactive surfaces and are commonly present in food production facilities, with air-filters and packaging machines prime areas for *Cronobacter* to reside [6]. The World Health Organisation (WHO) risk assessment indicate that *Salmonella* serovars and *Cronobacter* species are the only bacterial pathogens which provide clear evidence of associated fatalities [7]. Strict manufacture guidelines are the current control method to reduce *Cronobacter* contamination, with environmental monitoring of Enterobacteriaceae a legal requirement in the production of PIF [8].

Cronobacter species cause bacteraemia, necrotizing enterocolitis, meningitis, and sepsis in neonates and infants, and wound or urinary tract infections in adult populations [9]. Occurring globally, *Cronobacter* species have been connected to recent outbreaks across Europe, Africa, and North America [10-12], however the number of *Cronobacter* infections documented is most likely underestimated as it is not a notifiable disease. Nonetheless, eight countries reported cases of *C. sakazakii* infections between the years of 1958 and 2016 [13]. Harvard database website has a summary of *Cronobacter* outbreaks in neonates [14] available publicly at https://doi.org/10.7910/DVN/TZ5PV9 (accessed June 2022). Regarding adult infections, Hayashi *et al.,* [15] recently described the case of a

69-year-old male who was admitted to the emergency department with a urinary tract infection and subsequently went into septic shock. *C. sakazakii* was isolated from their blood and recorded as the causative agent. Interestingly, the patient had no underlying conditions. The authors state that *Cronobacter* species should be recognised as a potential pathogen in non-immunocompromised adult populations [15].

To reach systemic persistence in the susceptible human host, virulence mechanisms are initially employed in the critical stages of environmental endurance and preliminary infection strategy. As an enteric pathogen, the main entry route of *Cronobacter* species into the host is through ingestion of contaminated food. This pathogen displays a high tolerance for stressful environments and can produce biofilms, which increase survival in the built food manufacturing facility [6]. Following entry into the host, the infection strategy deployed is distinct, being composed of the subsequent stages: adhesion, colonisation, invasion and translocation [16]. *Cronobacte*r species employ a range of virulence mechanisms and can successfully evade the immune system [9]. Regarding chemotherapeutic treatment, broadly these bacteria remain susceptible to compounds such as β-lactam antimicrobials, however they have since gained resistance to several antimicrobials [17].

This review explores the molecular features of *Cronobacter* species which support their environmental persistence including high stress tolerance, biofilm formation, efflux pumps and development of subpopulations; the mechanisms involved in infection strategy encompassing secretion systems, quorum sensing, colonisation and translocation; the actions which aid survival in the host such as competitive acquisition and utilisation of minerals and metabolites respectively, in tandem with immune system evasion and AMR. The rationale for this review is to provide an overall insight into the pathogenic mechanisms utilised by *Cronobacter* during 3 key stages (figure 1), to highlight gaps in current knowledge of this food-borne pathogen of global concern.

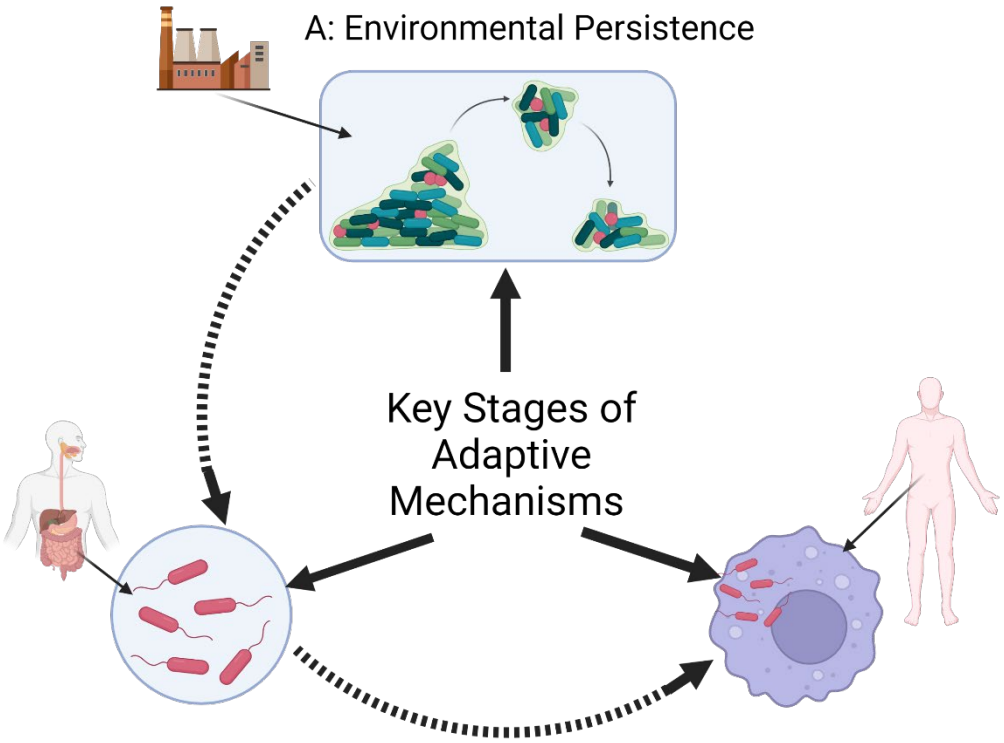

Figure 1: Part A: **Environmental persistence** of *Cronobacter* species in the food manufacture setting is a key stage which allows entry into the food chain. Biofilm formation, depicted here, along with adaptations to high temperature, acidic conditions and osmotic pressure support this persistence.

Part B: **Infection strategy** of *Cronobacter* species occurs following ingestion of contaminated food or water. Mechanisms in this stage of virulence include secretion, motility, quorum sensing, colonisation, and translocation.

Part C: **Systemic persistence** in the host is the ultimate virulence stage of *Cronobacter* species. Evasion of the immune system, specifically survival and replication inside the human macrophage is depicted. Image created with BioRender.com.

2. Detection of *Cronobacter* species in different environments

Cronobacter species have been recovered from a range of environments as shown in table 1, yet their natural habitat is not definitively known. Based on characteristics including solubilization of mineral phosphate and siderophore production, which are plant-associated traits [18], literature initially suggested that plants could be the indigenous environment of *Cronobacter* species, particularly *C. muytjensii* and *C. dublinesis* [4].

However, the natural habitat of *Cronobacter* species cannot be narrowed to one specific niche, with environments including water, soil, vegetables, and dried food reported as primary sources [19].

Table 1: Different foods and environments where *Cronobacter* species were recorded recently.

Location	Source	Location	Prevalence	Year & Reference
Food	Aquatic products- Fish & Shrimp	China	31/800 samples, 3.9%	2020 [20]
	Infant cereals	Brazil	13/75 samples, 17.33%	2020 [21]
	Meat & meat products	Guangdong, China	54/588 samples 9.18%	2020 [22]
	Powdered infant milk	Tehran City, Iran	25/364 samples, 6.86%	2022 [23]
	Goat milk-based powered infant formula	Shaanxi Province, China	32/750 samples, 4.27%	2022 [24]
Environments	Milk powder manufacturing facility	Serbia	15/100 samples, 15%	2021 [25]
	Milk powder manufacturing facilities	Maryland, USA	2495/5671 samples, 44.4%	2020 [26]
	Neonatal sepsis	Egypt	12/100 samples, 12%	2020 [11]
	Human clinical stool samples	Wenzhou, China	12/1024 samples, 1.17%	2020 [27]
	Filth flies	USA, Europe, Southeast Asia	19 strains/14 flies	2020 [28]

While *Cronobacter* species is commonly associated with processed milk products such as powdered milk, it is not present in fresh raw milk [29]. Constituents of fresh milk have been adapted to inhibit this pathogen [30], yet the components of fresh raw milk which naturally inhibit *Cronobacter* species remain to be elucidated. Nonetheless, this bacterium can occupy specific environmental niches, in which competition for resources is low due to the harsh nature of the conditions.

3. Environmental Persistence

Extreme drought, high temperatures and low pH is tolerated by adaptable species of the *Cronobacter* genus and supported by a variety of molecular features and mechanisms as outlined in figure 2.

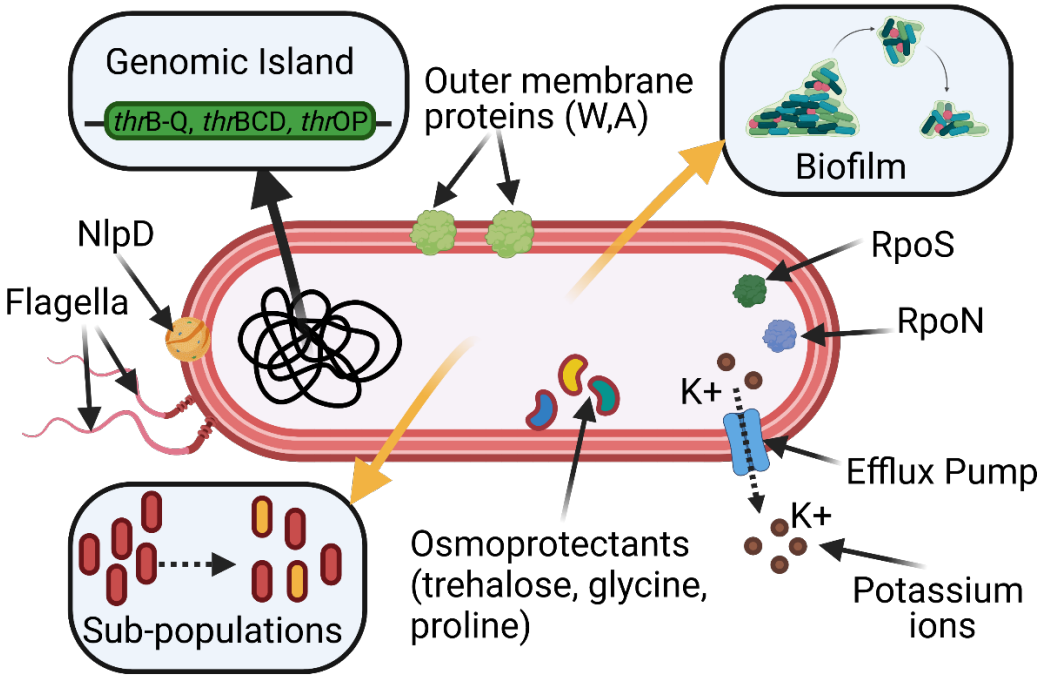

Figure 2: The molecular features of *Cronobacter* species which support mechanisms involved in environmental persistence. Outer membrane proteins W and A facilitate adaptation to osmotic pressure and biofilm formation respectively. RpoN and RpoS are stress response sigma factors involved in osmotic pressure and acid tolerances respectively. Efflux pumps transport potassium ions out of the cell, and along with osmoprotectants, aid in desiccation tolerance. Thermotolerance is supported by *thr* genes, and the lipoprotein NlpD facilitates acid tolerance. Orange arrows indicate mechanisms. Image created with BioRender.com

3.1 Adaptation to High Temperature

Cronobacter species, one of the most heat tolerant members of the Enterobacteriaceae family, can be inactivated upon exposure to 70^0C [31]. In the process of rehydrating infant formula, it is imperative to reach minimum temperature of 70^0C to reduce the risk of

infection in infants, and its associated morbidity and mortality [20]. Variations in the rehydration methods and failure to reach the critical value of 70^0C may provide *Cronobacter* the opportunity to establish infection following ingestion. Furthermore, bacterial resistance to lethal conditions can be enhanced by exposure to sublethal doses, which encourages the bacteria to respond by undergoing genetic variation or physiological adaptation [31]. This phenomenon is known as cross-protection whereby bacteria adapt to a harsh environment by pre-conditioning with a sublethal exposure of a different stressor. *C. sakazakii* displayed this ability in a study by Yang *et al*. [28], in which the pathogen underwent heat-shock and was exposed to environmental stresses such as heat, acid or desiccation. Improved tolerance to these environmental stressors was reported when compared to the untreated isolates [31].

Numerous factors can influence the tolerance of *Cronobacter* to heat, these include the varying properties of the food or formula matrix, such as high lipid content or low water activity [32]. Presence of a specific genomic island containing *thr*B-Q, *thr*BCD and *thr*OP genes, has been suggested to support augmented thermotolerance in *Cronobacter* species. Orieskova *et al*. [33] reported 49/73 *C. sakazakii*, 9/14 *C. malonaticus* and 16/17 clinical isolates of both species contained this genomic island. Survival at 58°C was significantly greater in those containing the genomic island when compared to a *C. sakazakii* mutant lacking the island [33].

3.2 Adaptation to Osmotic Pressure and Desiccation

Fluctuations in osmolarity can cause significant damage to a bacterial cell, yet *Cronobacter* species display a higher tolerance to both desiccation and osmotic pressure compared to other food-borne pathogens including *Salmonella* species and *Escherichia coli* [34]. Interestingly, *Cronobacter* species grown and dried in PIF showed higher survival rates than when grown and dried in tryptic soy broth, suggesting a role for PIF components in desiccation tolerance [35].

Several genes and mechanisms involved in tolerance to desiccation have been identified (table 2) including the genes for the transport of osmoprotectants glycine, betaine, and

trehalose [36]. Production of trehalose, glycine and proline coupled with the accumulation of metal ions support water membrane maintenance in *Cronobacter* species and thus may aid in tolerance to desiccation. Other genes have been identified as crucial for adaptation to both osmotic pressure and desiccation (table 2), yet the exact mechanisms of some remain to be fully elucidated.

Table 2: Summary of virulence characteristics instrumental in adaptation to osmotic pressure and desiccation by *Cronobacter* species (see also figure 2)

Virulence factor	Mechanism	Reported in:	Reference
RpoS	Response sigma factor involved in response to hyperosmotic conditions	*C. sakazakii*	2016 [33]
Cpx system	Regulator of envelope stress response	*C. sakazakii*	2014 [37]
RpoN	Response sigma factor involved in response to hyperosmotic conditions	*C. sakazakii*	2014 [37]
OpuC & ProP systems	Transport of osmoprotectants glycine, betaine, and trehalose	*C. sakazakii*	2019 [38]
Outer membrane protein W (OmpW)	Involved in response to osmotic pressure	*C. sakazakii*	2018 [39]
YqiF	Inner membrane protein, supports osmotic stress response, exact mechanism unreported	*C. malonaticus*	2018 [40]
KafA	Potassium efflux protein, supports osmotic stress response	*C. malonaticus*	2018 [40]
Peptidylprolyl isomerase	Supports osmotic stress response, exact mechanism unreported	*C. malonaticus*	2018 [40]
Cys-tRNA deacylase	Supports osmotic stress response, exact mechanism unreported	*C. malonaticus*	2018 [40]
Oligolacturonate lyase	Supports osmotic stress response, exact mechanism unreported	*C. malonaticus*	2018 [40]

3.3 Adaptation to Acidic Environments

Enteric pathogens face numerous challenges in the host environment, including the extremely acidic conditions of the stomach. While the pH of adult stomachs range from 1.5 to 3.5, the stomach pH of a 28-day old baby can be as high as 5.17 [41], allowing enteric pathogens such as *C. sakazakii* a greater chance at survival. *Cronobacter* species

have a reported tolerance for acidic conditions, with the level of tolerance dependent on the species and the type of acid. In a laboratory setting, *Cronobacter* species can tolerate acidic conditions as low as pH 4.2, and good bacterial growth is reported in media with PIF at pH 5 [37]. Research by Tong *et al.* [42] involved a transposon mutagenesis approach to elucidate the factors associated with the acid tolerance of *C. malonaticus*. Eight mutation sites including those of glucan biosynthesis protein G, extracellular serine protease, nitrogen regulation protein NR (II), lysine transporter, glucosyltransferase MdoH, phosphate transporter permease subunit PstC, sulfate transporter and D-alanine-D-alanine ligase produced mutants with lower acid tolerances. The biofilm of each mutant was much reduced in terms of mass, and subsequently, when grown at pH 4, all mutants displayed lower cell viability and immature biofilms compared to the wild type (WT) [42].

Using a similar transposon mutagenesis approach, Alvarez-Ordonez *et al*. [43] identified *rpoS* was associated with a higher acid tolerance in *C. sakazakii*. Furthermore, Alvarez-Ordonez *et al.* [37] report the critical role of the envelope stress response regular CpxR which senses differences in the environment and thus regulates expression of genes to enhance membrane integrity. Ji *et al.* [44] compared *nlpD* mutants to the WT *C. sakazakii* and reported that *nlpD* encodes an acid-resistance factor, with mutants exhibiting attenuated virulence. Lipoprotein NlpD, located in *C. sakazakii* outer membrane, is reported to function in the response to acidic stress, as well as in maintenance of membrane integrity. This conserved lipoprotein is suggested to be a viable drug target as *nlpD* is a novel virulence factor and NlpD is located on the outer membrane of the bacterial cell [44].

3.4 Biofilm Formation

Biofilms are a key virulence factor of pathogenic bacteria and are often deployed as a defence mechanism against environmental stressors including high temperature, osmotic pressure, or acidic conditions [45]. Environmental factors can have an impact on biofilm formation by *C. sakazakii*, as outlined by Jung *et al.* [46], who report that media, sucrose concentration and storage humidity affected the production of exopolysaccharides (EPS)

and the subsequent formation of biofilm. Current standard procedures of cleaning and sterilization may be insufficient at removing established biofilms, and may actually be aiding the dispersal of bacteria, promoting subsequent biofilm formation at distant sites [47].

Bacterial cellulose is the main constituent of EPS [48] and *bcsG* has been identified as the main gene encoding for cellulose biosynthesis in *Cronobacter* species [49]. Together with flagellum forming genes *flgJ, flhE, fliD, flhD* and the outer membrane protein A (OmpA) and OmpW, the cellulose-related genes influence biofilm formation in *Cronobacter* species. Of particular importance is *bcsG,* as it is involved in regulating phosphoethanolamine head transfer from phospholipids, thereby ensuring maintenance of membrane protein production [49]. The *bcsG* gene interacts with *bcsA*, ensuring bcsA becomes integrated into the cytoplasmic membrane [50]. Li *et al.* [49] analysed the capability of six *Cronobacter* species to form biofilms and deduced that *bcsG* influenced biofilm formation in strain DSM 18707. EPS levels were significantly reduced in the *bcsG* knockout, suggesting its pivotal role in positively regulating EPS and biofilm formation [49]. The negative regulator of cellulose biosynthesis, *bcsR,* is shown to have an impact on biofilm formation. Gao *et al*. [51] detected a 50% decrease in biofilm formation in a *bcsR* mutant when compared to its isogenic WT. Cellulose expression was increased, biomolecules such as carotinoids, fatty acids and amides were significantly reduced, in addition to reduced expression of flagella and OMPs [51]. Ye *et al.* [39] found that mutants lacking OmpW had increased biofilm formation.

While the cellulose related genes and the OMPs certainly impact biofilm formation in *Cronobacter* species, the specific interactions of the cellulose biosynthesis genes, the flagellum forming genes and the OMPs require further research.

3.5 Other Mechanisms which Support Environmental Persistence

C. sakazakii is reported to persist for at least 4 years in PIF manufacture environment [52], with cellular mechanisms such as development of subpopulations and use of efflux systems supporting this persistence.

Efflux pumps are reported to aid persistence in stressful environments. Species specific efflux pump orthologues were reported in each of the seven species of *Cronobacter*, with the acquisition of these genes suggested to be either a result of an independent evolutional event or a microevolutionary selective event [53]. In extreme environments, key physiological responses of *Cronobacter* species are initiated and include the efflux of ions and the accumulation of sugars, like trehalose, in order to preserve cellular integrity [53]. When exposed to desiccation, the immediate response is to increase the internal osmotic pressure of the cell by augmenting the cellular content of potassium glutamate. The secondary response is to replace it with other osmoprotectants such as trehalose or proline that are more osmotolerant molecules than potassium glutamate [53]. In sum, efflux pumps aid cell buffering against harsh environments by influencing the internal conditions, and so are instrumental in the persistence of *Cronobacter* species.

Viable but non culturable (VBNC) cells are nonculturable on media which would normally promote bacterial growth but display viable characteristics upon examination and in favourable condirions, can return to a culturable, metabolically active state [54]. *C. sakazakii* can induce a VBNC state as a survival strategy, in response to antimicrobial pressure, with ampicillin reported to promote the switch to a VBNC state [55]. Interestingly, VBNC *C. sakazakii* displayed4.7 times higher survival rate in macrophages when compared to culturable cells [56]. Upon further examination, genes for OmpW and Superoxide Dismutase (SOD) were significantly upregulated in the VBNC population in contrast to the culturable one. OmpW expression relates to environmental stress while SOD is an enzyme capable of neutralising the reactive oxygen species (ROS) generated by the host as a defence mechanism [57]. Persister cells are another example of a subpopulation and display temporary antimicrobial-tolerance as the cells are in a state of dormancy, however whether *Cronobacter* species form persister cells remains undocumented.

4. Infection Strategy

Cronobacter employ several virulence mechanisms in its infection strategy (figure 3). Secretion systems and quorum sensing allows them to influence their surrounding environment, while motility, colonisation, and translocation enable them to migrate and colonise different tissues of the host.

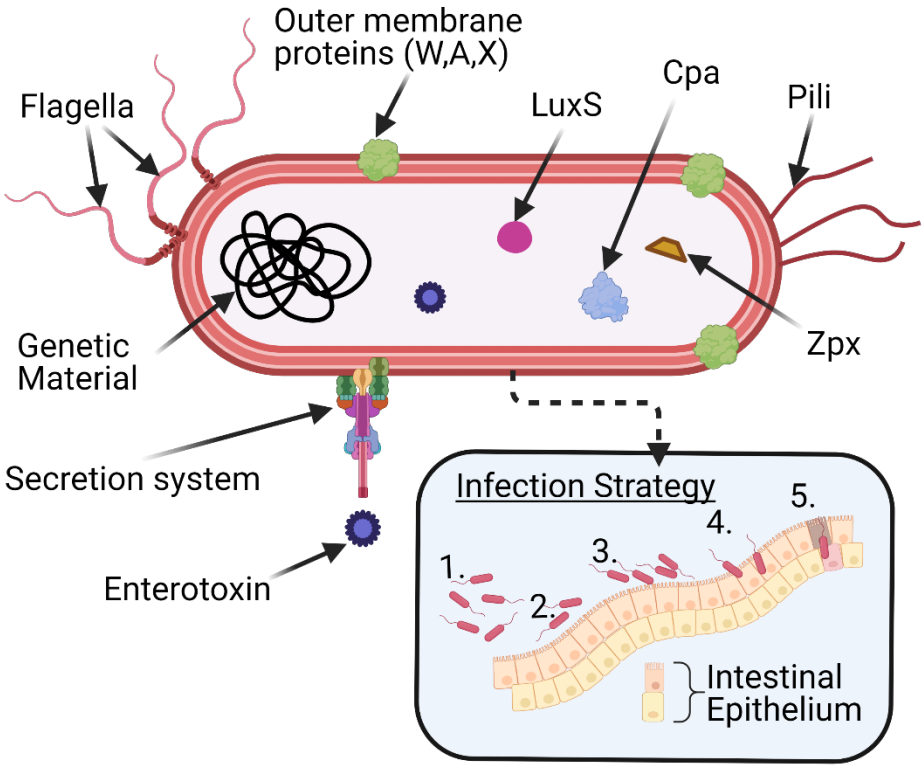

Figure 3: The molecular features of *Cronobacter* species which support infection of the host. Flagella and pili facilitate motility and adhesion to cell wall, with OMPs W, A and X being instrumental in invasion of host cells. Secretion of effector proteins such as Cpa, Zpx, and enterotoxin is supported by secretion systems. The LuxS protein is the key molecule in quorum sensing signal AL-2 synthesis. The inset depicts stages of the infection strategy as follows: 1. Entry, 2. Adhesion, 3. Colonisation, 4. Invasion into host cell and 5. Translocation through cell tissue. Image created with BioRender.com

4.1 Secretion of Effector Molecules

Bacteria secrete molecules such as effector proteins, small molecules, and DNA that elicit an immune response from the host and increases bacterial invasiveness [58]. Secretion systems (SS) are used to transport molecules such as proteins from inside the bacterial

cell directly to the host cells or to the extracellular fluid. *Cronobacter* species utilise type 1 secretion system (T1SS), T2SS, T4SS, T5SS and T6SS which are stringently regulated [9].

Cronobacter species secrete effector proteins, as toxins or exoproteins, that potentially elicite an immune response from the host cell [59]. Kucerova *et al.* [60] performed whole genome sequencing of *C. sakazaii* BAA-894, identifying plasmid pESA3 which was reported to encode an outer membrane protease with a high level of homology to the omptin family. Members of the Enterobacteriaceae family express omptins as OMPs which function as proteases, invasins or adhesins. This omptin-like protease is now referred to as *Cronobacter* plasminogen activator (Cpa) and closely identifies with the Pla subfamily of omptins [61]. It has been proposed that when *C. sakazakii* is invading the circulatory system of a host, and expresses Cpa, a complex with plasminogen is formed causing proteolysis and conversion of host plasminogen to plasmin [62]. Bacterial cells with plasmin bound to the surface can catalyse degradation of fibrin polymers, which are the end products of blood clotting. Furthermore, Cpa inactivates α2-anti-plasmin, which is responsible for plasmin degradation under normal circumstances [62]. Thus, activated plasmin results in increased degradation of fibrin polymers and therefore blood clots, leading to increased invasiveness and systemic spread of *C. sakazakii*.

Kothary *et al*. [63] report a zinc containing metalloprotease, Zpx, in *Cronobacter* species and identified that Zpx induced rounding of Chinese Hamster Ovary (CHO) cells, leading to damaged cellular membranes. *Cronobacter* species are also reported to produce enterotoxin which causes host cells rounding and lysis [32]. The toxin was shown to withstand holding temperatures of 50°C and 70°C and remained stable after 30 minutes at 90°C [32], therefore indicating that the enterotoxin is resistant to common pasteurization methods of 72ºC. In sum, *Cronobacter* uses different SS to release a range of effector proteins which cause proteolysis and increased invasiveness.

4.2 Motility

Cronobacter species are motile with adhesion factors, including pili and OMPs, that are common among all strains and essential for infection of the host cells, specifically adhesion, invasion, and biofilm formation [9].

To examine the effect of different protein expression on the ability of *C. sakazakii* to adhere and invade mouse neuroblastoma N1E-115 cells, Veronica *et al.* [64] performed a comparative genomic study of *C. sakazakii* ATCC BAA-894 and a *fliF*: Tn*5* nonmotile mutant. From proteomic analysis, 443 proteins were identified, with 361 common to both strains, 37 exclusive to the WT and 45 exclusive to the mutant [64]. The WT-associated proteins functioned mainly in motility, while the mutant exclusive proteins were involved in transport of lipids, carbohydrates, nucleotides, and enzymes. It also displayed a higher number of metabolism related proteins comparatively to the WT. Regarding gene analysis, although *cpa* was detected in both, WT had a higher abundance of this gene. However, the direct correlation between levels of *cpa* and motility remains to be completely characterised. In terms of adhesion, the mutant showed increased rates, while the WT displayed a higher rate of invasion of the mouse neuroblastoma cells. The study indicates that flagella support the invasion of cells, while a lack of flagella augments the rate of adhesion [64].

Flagella of *C. sakazakii* can stimulate proinflammatory response in the human host through the signalling pathway of toll-like receptor 5 [64]. Ling *et al.* [47] report that the knockout of *fliC,* which codes for a flagellar filament structural protein, results in decreased biofilm formation, adhesion, and motility in *C. malonaticus*. The reduced motility of flagellum-lacking mutants is reported by both research groups, however, Veronica *et al.* [64] document increased adhesion of *C. sakazakii* to mouse neuroblastoma cells while Ling *et al.* [47] report reduced adhesion of *C. malonaticus* to IEC-6, an intestinal epithelioid cell line.

Further studies can help clarify this variability between different bacterial species and response to different host cells. Nonetheless, it is clear that *Cronobacter* species flagella

are instrumental in many infection mechanisms including motility, adhesion, invasion, and biofilm formation.

4.3 Quorum Sensing

Li *et al.* [65] performed a comparative proteomic analysis of the adhesion and invasion related proteins of *C. sakazakii* using data-independent acquisition coupled with liquid chromatography-mass spectrometry (LC-MS). Upon comparison of two strains of *C. sakazakii* which differed only in terms of adhesion/invasion abilities, it was found that LuxS protein, the key molecule for the synthesis of quorum sensing signal AL-2, was expressed 3.55-fold higher in the strongly adhesive/invasive strain. This result suggests the potential role of LuxS protein in the adhesion and invasion of *C. sakazakii* [65]. To investigate the function of LuxS in biofilm formation, adhesion, and invasion, Ling *et al.* [47] performed a comparative analysis of a WT *C. malonaticus* and a *luxS* mutant. Increased expression of the flagellin negative regulator *flgM* was detected in the mutant when compared to the WT, indicating that LuxS directly impacts flagellar development and thus the adhesion and invasion capability of *C. malonaticus* [47]. The exact mechanism of action of LuxS protein on *Cronobacter* species adhesive/invasive ability remains to be fully characterised.

4.4 Colonisation and Translocation

Following entry and adhesion, the next step in the infection strategy is colonisation of host environments, such as the gastrointestinal tract (GIT). Virulence mechanisms aid colonisation of mucosal surfaces and facilitate translocation into the blood stream resulting in systemic infections such as sepsis [61]. One function of the GIT mucosal surface is to act as a barrier, limiting the adhesion and colonisation of pathogenic or commensal bacteria. The GIT mucosal layer is composed of glycoproteins such as mucins, antimicrobial peptides, digestive enzymes, and immunoglobulins [66]. The ability to colonise GIT and outcompete the resident gut microbiome for nutrients is a highly conserved phenotype which is expressed across the *Cronobacter* species [9].

Regarding colonisation, adhesion factors, including pili and OMPs are common to all strains of *Cronobacter* species and are essential for adhesion, invasion, and biofilm formation [9]. A study by Kim *et al.,* showed that OmpA and OmpW were required for both GIT apical and basolateral invasion by *C. sakazakii* [67]. Furthermore, a *C. sakazakii* OmpX deletion mutant, displayed decreased invasion of the human colonic carcinoma epithelial (Caco-2) cells and lower levels of colonisation in the liver and spleen of rat pups compared to the WT [67].

Following colonisation, *Cronobacter* species can initiate pathogenesis in the underlying tissues and the blood stream *via* translocation. Clinical *C. sakazakii* have been reported to translocate through Caco-2 and human brain microvascular endothelial cells (HBMEC) paracellularly, with certain strains of *C. sakazakii* showing higher invasion and translocation capability [68]. Variation between the transepithelial electrical resistance (TEER) of these cell lines were associated with a higher level of translocation by the bacterium, suggesting that clinical isolates of *C. sakazakii* translocate paracellularly by altering the host cells tight junctions to migrate between them [68]. This theory is supported by the increased invasion rates in individuals with impaired or under-developed tight junctions [17].

Molecular features of *Cronobacter* species which support colonisation are reported, however, the virulence factors unique to *C. sakazakii* clinical isolates with higher translocation capabilities remain to be fully outlined.

5. Systemic Persistence in the Human Host

Once established in the host, *Cronobacter* species can persist due to a combination of molecular features and virulence mechanisms (Figure 4) encompassing iron acquisition, sialic acid utilisation, AMR, and immune system evasion.

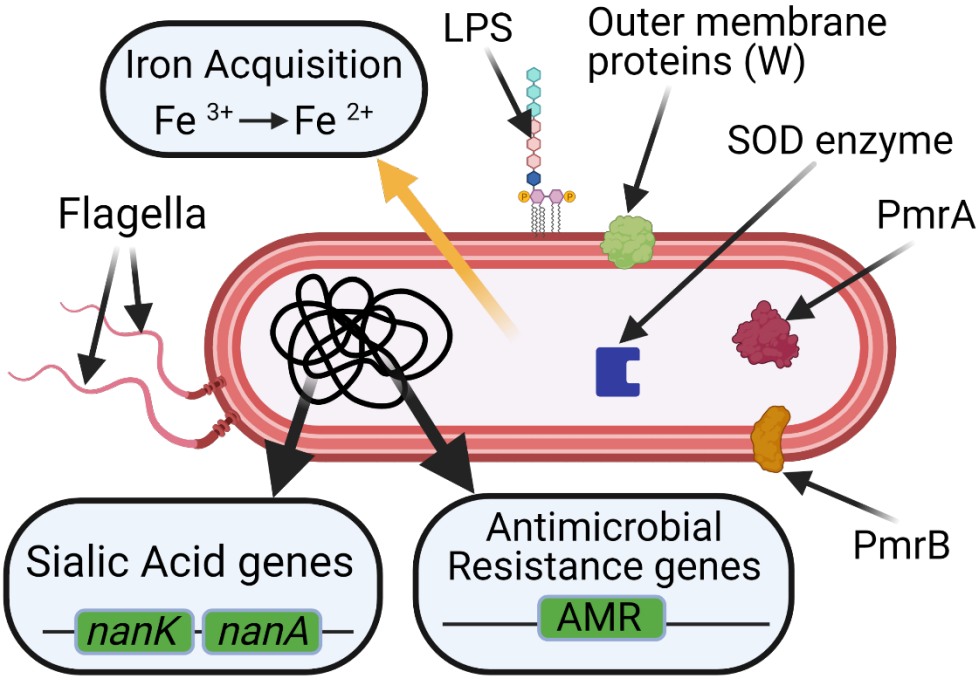

Figure 4: Molecular features of *Cronobacter* species which support virulence mechanisms involved in systemic persistence in the human host. Evasion of the immune system is facilitated by modification of the LPS, upregulation of OmpW, SOD enzyme activity and interactions of the two-component system PmrA/PmrB. Iron acquisition involves the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Sialic acid genes *nanK* and *nanA* allow the utilization of sialic acid in the host, while AMR genes convey resistance to a variety of treatment options. Orange arrow indicates a mechanism. Image created with BioRender.com.

5.1 Host Cell Defense

As an enteric pathogen, the predominant entry route of *Cronobacter* species is through ingestion of contaminated food or water. While the human body has many defence mechanisms against bacterial cells, *Cronobacter* species have adapted to evade those host defences like antimicrobial peptides in serum, and macrophage attack [68], among others. The intestinal epithelium is a critical protective barrier against food-borne bacterial infections, and if this layer is penetrated, the pathogen may gain access to the underlying tissues [66].

The pathogenicity of *C. sakazakii* is such that it can invade not only the intestinal epithelium but also the brain endothelium of hosts by crossing the blood-brain barrier (BBB) *via* adhesion and invasion of these host epithelial cells, including HBMEC [17]. HBMEC comprise the human BBB, which when compromised by *C. sakazakii* can cause meningitis, a potentially fatal infection in populations with limited or impaired immune response, for example neonates and infants [17]. Several cell lines, such as Caco-2, have been utilised to show these mechanisms of adhesion, invasion and translocation employed by *Cronobacter* species [67-72].

5.2 Evasion of Host Immune System

Cronobacter have evolved to evade the host's immune system response, displaying the capability to survive within macrophages, and resistance to the bactericidal action of blood serum and the complement system. Almajed and Forsythe [68] revealed that 9 out of 10 *C. sakazakii* clinical isolates withstood human serum exposure, with the single outlier lacking the *cpa* gene. Townsend *et al.* [72] investigated the virulence of *Cronobacter* isolates from an outbreak in 1994 in a French neonatal intensive care unit, during which 17 neonates were infected, 3 with a fatal outcome. *Cronobacter* strains were shown to persist and replicate inside human U937 macrophages, which should normally destroy invading bacteria, with the majority of isolates surviving for approximately 48 hours [72]. Factors supporting survival inside macrophages included induction of VBNC state, upregulation of OmpW and SOD enzyme activity [55].

Another factor which impacts macrophage action against bacterial pathogens is the presence of lipid A on the bacterial membrane. Lipid A is the most active component of the lipopolysaccharide (LPS), located on the outer membrane. In the human host, lipid A can be recognized by the innate immune system toll-like receptor 4 once the microbe has breached the skin or the GIT mucosa, often resulting in inflammation [73]. Regarding impact of lipid A on phagocytosis by macrophages, Kim *et al*. [74], reported that deleting *labp*, a novel virulence factor that promotes lipid A biosynthesis by LpxA in *C. sakazakii*, reduced phagocytosis. Mutants which lacked *labp* showed a 50-fold reduction in phagocytosis by macrophages compared to the WT [74]. These mutants were also shown

to be defective in their ability to invade Caco-2 cells, presenting structural changes in the OMPs, flagella and LPS, thus compromising *C. sakazakii* cellular integrity and virulence [74].

Modification of lipid A by the Two Component System (TCS) PmrA/PmrB is employed by *C. sakazakii* to reduce lipid A recognition by the host innate immune system. TCSs are composed of two proteins, a sensor kinase, in this case PmrB, which receives external signals from the environment, and a response regulator, PmrA, that conveys a change in the bacterial cell. In weak acidic conditions (pH 5), phosphoethanolamine modification of lipid A occurs, contributing to increased resistance to cationic antimicrobial peptides produced by the host cells [75]. This increased resistance improves *C. sakazakii* ability to evade the immune system of human hosts. Reporting on the impact of *C. sakazakii* TCS PmrA/PmrB on invasion and survival in macrophages, Hua *et al*. [76] stated that *pmrA*-lacking mutants grew faster and displayed greater number of replicates when recovered from the macrophages in comparison to the WT. However, mutant cells overexpressing *pmrAB* grew slower compared to the isogenic WT and intracellular numbers were shown to decrease over time. This study reports that *pmrA* reduces invasion and replication inside the macrophage, and that PmrA/PmrB TCS in *C. sakazakii* is important for host cell invasion and intracellular replication ability.

Regarding host cell modulation, Mittal *et al.,* [77] showed that *C. sakazakii* targets the dendritic-specific intercellular adhesion molecule (ICAM) nonintegrin to promote immunosuppressive responses *via* modulation of host mitogen-activated protein kinases. Dendritic cells infected with *C. sakazakii* expressed low levels of proinflammatory cytokines, allowing the pathogen to evade the immune system [77].

Undoubtedly, evasion of the host immune system is a prime virulence mechanism for *Cronobacter* species. Further insights into these cellular and molecular mechanisms are needed.

5.3 Iron Acquisition

Iron acquisition is an essential virulence mechanism for pathogens as once inside the human host they must compete, with the host and with the commensal bacteria, for this metabolically restricted mineral. Iron is required for many enzymatic reactions, as an essential cofactor, both in primary and secondary metabolism. While present in sufficient amounts for microbial growth, iron exists as ferric iron (Fe^{3+}) in human hosts, which is biologically inaccessible to bacteria. Therefore, to combat iron starvation while in a human host, bacteria produce siderophores which form stable complexes with ferric iron [78], scavenging iron from the host to the bacterial cell. Fe^{3+}-siderophore complexes in Gram-negative bacteria are transported into the periplasm by TonB-dependant receptors and then moved to the cytoplasm *via* ABC transporters [79]. Iron is released when the complex is reduced from ferric to ferrous iron (Fe^{2+}) in the cytoplasm, with siderophore decomposition also a mechanism of iron release to the bacterial cell.

Iron acquisition systems Feo and Efe, encoded by *feoABC* and *efeUOB*, along with enterobactin-like siderophores can be found in each of the seven *Cronobacter* species, with plasmid-harbouring strains expressing cronobactin, the aerobactin-like siderophore, for ferric iron transport [79]. Furthermore, a ferric dicitrate transport system was reported in clinical isolates of *C. sakazakii* and *C. malonaticus* but not in the other 5 species, indicating its potential function as a virulence mechanism [79]. Thus, the differences in iron acquisition systems by different *Cronobacter* species may reflect the availability of iron within their respective environmental niche, for example plants, PIF manufacturing sites or the human host.

5.4 Sialic Acid Utilisation

Selective environmental pressure has contributed to gradual adaptations in the *Cronobacter* genus, however not all species display the same adaptations. Regarding adaption to human hosts, only *C. sakazakii* can import and catabolise exogenous sialic acid [9]. Sialic acid is present in infant formula, breast milk, brain gangliosides, and intestinal mucin and plays various roles in the human body including neural transmission.

In contrast to the other members of the *Cronobacter* genus, *C. sakazakii* carries the *nanA* and *nanK* genes which encode for the use of exogenous sialic acid as a source of carbon [37]. Interestingly, in *E. coli,* sialic acid is instrumental in surface modification *via* regulation of sialidase and adhesin expression or by inhibition of the *fimB* gene involved in epithelial cell adhesion and invasion [80]. Whether sialic acid plays a role in surface modification of *Cronobacter* remains to be elucidated.

5.5 Antimicrobial Resistance

The abuse of antimicrobial agents through prolonged usage, inappropriate dosing, and length of application are thought to result in the development of AMR in pathogens, including *Cronobacter* species. Previously, *Cronobacter* were reported to be susceptible to β-lactam antimicrobials, however strains have since become resistance to others, including neomycin, tetracycline, and trimethoprim [17]. A study performed by Parra-Flores *et al.* [5] examined a diarrheic haemorrhagic outbreak in Mexico in 2011, during which *C. sakazakii* and *Enterobacter* species were isolated from samples of PIF, infant fecal matter and home environment. These authors reported on the virulence and AMR of *C. sakazakii,* with four out of five isolates displaying resistance to cephalosporins [5].

In a different study by Li *et al*. [27] *Cronobacter* species were isolated from food and clinical samples in Wenzhou, China, between 2008 and 2018. The 90 isolates were identified using multi-locus sequence typing (MLST) analysis of the seven housekeeping genes of *Cronobacter*, with sequence types determined using the seven-digit allelic profile obtained. Antimicrobial susceptibility of these 90 isolates was elucidated for a panel of fourteen antimicrobial compounds, with 84% of isolates reported as sensitive or with intermediate resistance to all antimicrobials under study. 16% displayed resistance to multiple antimicrobial agents, with six isolates (6.6%) showing resistance to six antimicrobial compounds [27], which increases the challenge for physicians to treat the infections caused by these multidrug resistant (MDR) isolates [81].

Regarding AMR genes, Parra-Flores *et al.* [82] examined seven *C. sakazakii* strains recovered from PIF and recorded the presence of the same efflux genes (efflux pumps

are quite useful in AMR), along with the antimicrobial inactivation gene *ampH* and the fosfomycin resistance determinant, *glpT*, in all tested strains. These genes confer resistance to a variety of antimicrobial compounds including β-lactams, aminoglycosides, fluoroquinolones, and phosphonates. In the same study, authors reported that all strains carried the *marA* gene which regulates multidrug efflux and membrane permeability [82]. In line with this study, Lepuschitz *et al.* [10] documented the presence of efflux genes, regulatory genes for modulation of antimicrobial efflux, *msrB,* an antimicrobial target protection gene, and *glpT* in *C. sakazakii*. Fosfomycin is viewed as an effective treatment against MDR bacteria, however both these studies confirm the presence of the *glpT* gene in *C. sakazakii* thus rendering treatment with fosfomycin ineffective.

Strains of *Cronobacter* have been isolated from both food and clinical environments worldwide, with the majority of isolates containing AMR genes such as *ampH, glpT, marA* and *msrB.* Screening of more clinical isolates for novel AMR genes is critical to monitor the rising resistance across *Cronobacter* species, and indeed all bacteria.

6. Mitigation Measures Against Infection

Currently, antimicrobial therapy is the only available treatment for infections, including those caused by *Cronobacter* species [83]. Suggested options include the broad-spectrum antimicrobials cephems and carbapenems, which inhibit cell wall synthesis, and quinolones, which inhibit DNA replication [84]. However, due to the rising prevalence of AMR among *Cronobacter* species [5:10:17:27:82], and indeed all bacteria, new treatment options are needed.

A variety of developmental *Cronobacter* species inhibitors, from different natural or synthetic origins, have been investigated (table 3), with targets including the virulence mechanisms and the general integrity of the cell membrane and wall. Biofilm formation, motility, secretion, adhesion, and translocation have all been targeted, with varying degrees of inhibition reported.

Table 3: Novel inhibitors of *Cronobacter* species, the cellular features and/or mechanisms compromised, and the overall impact on the cell.

Inhibitor type	Example(s)	Feature/ mechanism(s) compromised	Overall impact	Reference
Plant-derived	Citral	Motility, biofilm formation, quorum sensing, endotoxin production	Reduced virulence	2017 [3]
	Oregano	Cell wall & membrane	Inhibited growth	2020 [85]
	Amaranthus tricolour	Biofilm formation & cell membrane	Reduced virulence/ cell death*	2020 [86]
	Thymoquinone	Motility, secretion & biofilm formation	Reduced virulence	2017 [87]
Nanotechnology	Silver nanoparticles	Membrane integrity	Cell death	2018 [88]
Bacteriophage	vB_CtuP_A24	Cell wall & membrane	Cell death	2021 [19]
	GAP32	Cell wall & membrane	Cell death	2014 [89]
Milk protein	Sweet whey protein concentrate	Translocation & invasion	Reduced virulence	2016 [71]
	Buttermilk	Adhesion	Reduced virulence	2017 [90]
Biopolymer	Chitooligosaccharides	Biofilm formation	Reduced virulence	2019 [45]
Recombinant peptide	Funme peptide	Cell wall & membrane	Inhibited growth	2019 [91]

*dependent on concentration used

The most prevalent target for these potential novel control methods is the cell membrane integrity, with different compounds reported as effective inhibitors, such as the plant-derived citral and milk-derived antimicrobial peptides [3; 20; 90]. Cellulose could also be a viable target due to its participation in biofilm formation, however external cellulose can also be modified to act as a delivery system to transport inhibitors, such as oregano oil [85]. Other approaches include the use of silver nanoparticles [88], however host toxicity concerns are a limitation. Indeed, the generation of a novel control method that is both

effective against *Cronobacter* species and host-safe is a major challenge. Another challenge is the elucidation of virulence factors and mechanisms of infection employed by *Cronobacter* species, since potential novel approaches to fight these infections rely heavily on this knowledge.

7. Conclusion and Perspectives

Cronobacter species employ a multitude of mechanisms to aid their virulence throughout the three key stages of environmental endurance, infection strategy and systemic persistence in the host. Tolerance to high temperatures, acidic environments, desiccation, and osmotic pressure enable *Cronobacter* species to persist in harsh habitats, including manufacturing facilities for PIF. Actions of *C. sakazakii* which are instrumental in its infection strategy include the use of secretion systems, motility, quorum sensing, colonisation and translocation, similar to the broad spectrum of pathogens. Sialic acid utilization by *C. sakazakii* and the competitive acquisition of iron support *Cronobacter* persistence once inside the host, along with deployment of diverse immune system evasion techniques, like lipid A modification, and the acquisition of AMR genes.

The past decade has revealed many insightful mechanisms of virulence, but also many new questions that require further research to be answered. New genes and proteins have been discovered, and their impact on virulence phenotypes reported, however, in many cases the underlying mechanism of action is not yet fully characterized. Further insights might be gained through genome comparison of the more virulent *C. sakazakii* species with those considered less virulent, those that are plant associated, such as *C. dublinesis* and *C. muytjensii*. Additionally, use of updated disease models may aid in the elucidation of the exact mechanisms which govern the successful infection of a human host. More information is required on how these pathogens evade the human host immune system, and how they potentially modulate host cells.

In tandem with the insights into virulence mechanisms, numerous developmental methods to control *Cronobacter* species have been described in recent years. Many of these target virulence phenotypes such as adhesion and translocation, however no gold

standard control method exist for the food industry as of yet. Increased knowledge of the pathogenic mechanisms of *C. sakazakii* can support the development of efficacious and consumer safe control methods for use in the food manufacture setting. This pathogen of global concern must be a priority for the benefit of food safety, food industry and the general public.

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