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Review Article

Cronobacter sakazakii (*Enterobacter sakazakii*): An Emerging Food borne Pathogen

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Abstract

Cronobacter sakazakii (*Enterobacter sakazakii*) is an emerging pathogen that causes necrotizing enterocolitis, sepsis, and meningitis. This bacterium is opportunistic pathogen and is linked with life-threatening infections in neonates and elderly persons. It is considered as ubiquitous organism and can be found in a broad range of foods & food ingredients and in water, in a variety of areas, including hospitals and houses though outbreaks most commonly associated with the ingestion of contaminated powdered infant formula (PIF). International Commission on Microbiological Specifications for Foods has ranked *C. sakazakii* a "severe hazard for restricted populations." It poses high tolerance to environmental stresses such as osmotic stress, elevated temperature etc. and decontamination processes and has broad antibiotic resistance and resistance to bile salts and disinfectants. *Cronobacter sakazakii* may survive in macrophage cells and efficiently attach to and invade epithelial cell lines, produce exopolysaccharide, form biofilm and has active efflux pumps. Controlling the organism in the production environment, thereby reducing dissemination, necessitates the provision of suitable diagnostic tools. Appropriate measures by parents, food and infant formula manufacturers, and health care providers, as well as understanding of the pathogenesis, are important in the prevention of *C. sakazakii*-related infections.

Keywords: *Cronobacter*, infant formula, virulence, pathogenecity

1. Introduction

Cronobacter spp. are opportunistic food borne pathogens associated with infections in neonates and infants, particularly those that are premature or immune-compromised[1]. Symptoms of *Cronobacter* infection are severe, including meningitis, septicemia and necrotizing enterocolitis[2,3]. The frequency of *C. sakazakii* infections appears to be low, prognosis is poor. Reported case-mortality rates vary from 40% to 80% among infected infants[4]. Low contamination levels (1 cfu.100 g^{-1}) of *Cronobacter sakazakii* can have a severe impact on health and the rapid detection and correct identification of these pathogens are important for food safety[3]. Only *Cronobacter* species associated with neonatal

infections, namely *C. sakazakii, C. malonaticus* and *C. turicensis* have the genes encoding for a cation efflux system which allows bacteria to invade brain micro vascular endothelial cells. As it is still unclear whether all of the species are virulent, the genus is currently classified as pathogenic. However, infant formula milk (IFM) is the only source that has been epidemiologically linked to disease outbreaks caused by *Cronobacter*[3,5,6,7]. The risk of *Cronobacter* contamination is further increased as it has been reported that regularly used disinfectants are insufficient to kill *Cronobacter* cells imbedded in biofilms[8]. Case-reports of *C. sakazakii*- infections in adults are rarely published because of the less severe nature of the illness in comparison with the high lethality of *C. sakazakii*- infections occurring in new-born infants[9]. Oral and intestinal colonization with *C. sakazakii*[10,11] may be associated with ingestion of contaminated food. The original reservoir of *Cronobacter* is still unknown but there are indications that these pathogens might be of plant origin.

Cronobacter strains have been isolated from various food products such as mixed salad vegetables, meat, milk and cheese[12,13,14,15,16]. Food other than infant formula has been rarely investigated for the presence of *C. sakazakii.* Nevertheless, this microorganism could be isolated from a wide spectrum of food and food ingredients. Although *C. sakazakii* contaminated food do not have general public health significance, measures for prevention should consider the presence of *C. sakazakii* in food, food ingredients, their processing and preparation as possible source of contamination, colonization or infection. Farmer *et al*.[10] recommended that presumptive *Cronobacter* isolates could be tested for susceptibility to the antibiotics cephalothin and ampicillin. These researchers found that 13% of *Cronobacter* isolates were susceptible to cephalothin and that all the isolates tested were susceptible to ampicillin. Other studies have confirmed the susceptibility of *Cronobacter* to ampicillin[17,18,19]. Ampicillin resistance in *Cronobacter* has, however, also been reported[9,20]. It was suggested by Lai[9] that this resistance was likely due to the overall increasing trend of antibiotic resistance in members of the *Enterobacteriaceae* in general. It has been determined that API 20E biochemical kits are not reliable or consistent tools for the correct identification of *Cronobacter* strains[21,22]. The polymerase chain reaction (PCR) has been shown to be a more reliable approach to identifying *Cronobacter* in comparison with the conventional identification techniques[23,24].

2. The Organism- *Cronobacter sakazakii*

Cronobacter sakazakii is a Gram-negative, motile organism and a member of the family Enterobacteriaceae, genus *Enterobacter* that is 1 µm x 3 µm in size. This bacteria is facultative anaerobic and generally peritrichous. *Cronobacter sakazakii* is generally indole, dulcitol and malonate negative, but methyl-α-D glucopyranoside positive[22]. The activity of α-glucosidase has been implemented as a selective marker in differential chromogenic agar[25]. Species of this genus can grow at temperatures between 6-47 °C with an optimum growth temperature of 39 °C[13]. However, some strains are inhibited at temperatures above 44 °C[1, 25] and some strains are capable of growing at 5 °C[26]. Pigment production and colony size on tryptone soy agar (TSA) are greatly influenced by the incubation temperature. The yellow pigment production after 24 h of incubation is more pronounced at 25°C than at 36 °C, with colony sizes of 1 – 2 mm and 2 – 3 mm, respectively[10]. Two colony types have been observed when isolates were streaked on TSA. TypeA[10] or matt[13] is dry or mucoid, scalloped and rubbery when touched with a loop. Type B[10] or glossy[13] is smooth and often exhibits little pigment production. Sub-culturing of the different colony types showed that the matt colonies may spontaneously change to glossy colonies and it is very common to find both colony types in one culture[10]. Differences between environmental strains and clinical strains were also observed[15]. The clinical strain produced mucoidal colonies on violet red bile glucose agar (VRBGA), whereas the environmental strains produced crinkled, matt colonies with a rubbery texture. Similarly, a reported heteropolysaccharide capsule may facilitate survival of the bacterium throughout the long shelf-life of PIF (24 months), as well as attachment to surfaces and formation of biofilms, promoting resistance to cleaning agents and disinfectants[13, 19, 27].

3. Characteristic features of *C. sakazakii*

1. General features: Gram negative rods, facultative anaerobic, no spore forming, mesophilic, yellow pigmented, osmotolerant, generally motile, peritrichous, provided of capsule.
2. Maximum temperature of growth: 41-45 °C. Minimum temperature of growth: 5.5-8.0 °C. No growth at 4 °C.
3. Distinguished into 16 bio groups.
4. Generation times 40 min at 23 °C 4.18-5.52 h at 10 °C 75 min a 25 °C in reconstituted infant milk powder.
5. Significant biochemical features- Catalase positive, oxidase negative, alpha-glucosidase positive, phosphoamidase

negative.

6. Habitat- Foods, environment, animals, biological liquid.
7. Virulence factor- Enterotoxin.
8. Antimicrobial susceptibility- Tetracycline, aminoglycosides, beta-lactam antibiotics, chinolone, antifolates, chloramphenicol, nitrofurantoine.
9. Chemical compounds susceptibility- Chitosans, oligomers of chitosans, monocaprylin (caprylic acid ester)

4. Taxonomy

Cronobacter sakazakii previously referred to as 'yellow pigmented *E. cloacae'*, was defined as a new species in 1980[10] and 15 biogroups were described based on biochemical characterization. Members of this species were considered relatively phenotypically and genotypically heterogeneous[28], a 16th biogroup has been reported and the existence of several genetic groups has been demonstrated based on 16S rRNA gene sequence analysis[29]. In recent years, it has been identified as a distinct species and researchers named it in honor of the Japanese bacteriologist Riichi Sakazaki who greatly contributed in understanding *Enterobacteriaceae*[10]. *E. sakazakii* isolates were reclassified in the novel genus, *Cronobacter* in 2008. Currently there are five distinct species in the genus, namely *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter dublinensis* and *Cronobacter muytjensii*[22]. The strains of *C. sakazakii* are allocated in biogroups 1-4, 7, 8, 11 and 13[30]. *Cronobacter malonaticus* is the closest related to *C. sakazakii* of all the *Cronobacter* species[31] and is characterized by the utilization of malonate[32]. This species is negative for indole and dulcitol utilization and include biogroups 5, 9 and 14. *Cronobacter turicensis*is derived from biogroup 16 identified by Iversen *et al*.[29]. *Cronobacter muytjensii* is the only *Cronobacter* species that is negative for the utilisation of 1-0-methyl-α-D glucopyranoside. This species is also positive for indole, dulcitol and malonate utilisation. *Cronobacter muytjensii* consists of strains in biogroup 15[10]. Automated ribotyping and F-AFLP, however, showed these strains to be separate from the other *Cronobacter* species. *Cronobacter dublinensis* consists of three subspecies, namely *C. Dublinensis* subsp. *dublinensis*, *C. Dublinensis* subsp. *lausannensis* and *C. Dublinensis* subsp. *lactaridi*. *Cronobacter dublinensis* subsp. *dublinensis* contains strains from biogroup 6 and the type strain was isolated from an environmental sample in a milk processing facility. The strains from these three subspecies were grouped together with sequence analysis based on 16S rRNA, F-AFLP and ribotyping[22]. Based on these results the three biogroups were designated as three subspecies[33]. These subspecies are generally dulcitol negative and indole production is variable[32].

5. Phylogeny

The reclassification of *Enterobacter sakazakii* to *Cronobacter* was based on a polyphasic approach that included DNA-DNA hybridisation, amplified fragment length polymorphisms (AFLP), automated ribotyping, full length 16S rRNA gene sequencing and phenotypic analysis[22, 34]. A total of 210 strains, previously described as *Enterobacter sakazakii* were divided into 16 biogroups based on indole production, methyl red test, Voges-Proskauer, ornithine decarboxylase, motility, malonate utilisation and acid production from inositol, dulcitol and methylglucoside. Defining characteristics of each biogroup corresponded with previous findings and included indole, dulcitol and inositol tests[22, 29]. Sequence analysis based on 16S rRNA of these strains resulted in four clusters. The majority of strains were grouped in cluster 1together with the *Enterobacter sakazakii* type strain, ATCC 29544T. Automated ribotyping of the 210 strains resulted in four groups largely corresponding with the four 16S rRNA clusters. The ribotyping results showed a similarity pattern of less than 62 % between the *Enterobacter sakazakii* strains and other *Enterobacteriaceae*. Subsequent fluorescent-AFLP (f-AFLP) analysis divided the strains into 6 groups that corresponded with the 16S rRNA clusters, as clusters 1 and 2 were each divided into two groups[22]. DNA-DNA hybridisation is considered to be the "gold standard" method to evaluate relatedness between bacterial species[35]. The recommended cut-off point for species delineation is regarded at a DNA homology of more than or equal to 70 % between two strains[36]. Representative strains of each of the four 16S rRNA clusters were subjected to DNA-DNA hybridisation and these strains were divided into five groups which had DNA-homology values of less than 70 %. Based on the combination of the genetic and phenotypic data, four *Cronobacter* species were proposed, *Cronobacter sakazakii, Cronobacter turicensis, Cronobacter dublinensis* and *Cronobacter muytjensii,* an additional *Cronobacter* genomo species 1 and a subspecies namely *C. sakazakii* subsp. *malonaticus.* This subspecies grouped separately from *C. Sakazakii* strains with f-AFLP and ribotyping analysis, but had a 99.6 % similarity based on 16S rRNA with the *C. sakazakii* type strain, ATCC 29544T[22]. This subspecies was accepted as a distinct species, namely *Cronobacter malonaticus* after

subsequent DNA-DNA hybridisation indicated that the *C. malonaticus* strains had DNA homology values of less than 70 % with the other *Cronobacter* species[33]. There is a high level of similarity between *C. sakazakii* and *C. malonaticus* and sequence analysis based on 16S rRNA is not sufficient to distinguish between these two species[22,31,33]. Biochemical differentiation between the two species can be accomplished by testing for the utilisation of malonate, although a small number of *C. sakazakii* strains do utilize malonate[34]. Controversial results regarding these two species have been found when the phenotypic data of 150 isolates was compared to ribotyping results[36]. According to biochemical analysis strain 05CHPL02 was identified as *C. sakazakii* and strain 05CPL53 as *C. malonaticus*. However, the ribotyping results placed the *C. Sakazakii* strain closer to the non-sakazakii strains and the *C. malonaticus* strain was grouped with the *C. sakazakii* strains[37]. A higher resolution between *C. sakazakii* and *C. malonaticus* were obtained with multilocus sequence typing (MLST) based on seven genes. The strains of these two species were clearly phylogenetic distinct, supporting the organization of *C. sakazakii* and *C. malonaticus* in two distinct species[38].

6. Reservoirs of *C. sakazakii*

The natural habitat of *C. sakazakii* is currently unknown. *C. sakazakii* can be found in the environment and in foods, with the most probable sources of the latter being water, soil and vegetables. Based on some of the organism's physiological features, it is likely that its natural habitat is on plant material[39]. These physiological traits aid environmental survival, and include the ability to produce a yellow pigment that protects the cell against UV rays in sunlight, capsular and fimbriae formation to aid in adherence to surfaces including other cell types, and its ability to resist desiccation during long dry periods. This also explains why the organism is isolated from certain plant-related products and ingredients, including dry herbs and spices[25]. These characteristics may also lend themselves to aid the organism's persistence in the formula, manufacturing and domestic environments. Kandhai *et al*.[40] isolated *C. sakazakii* from almost all environments examined, including milk powder manufacturing facilities and household vacuum cleaners. In eight of the nine facilities, samples were positive, and 5 of the 16 households were also found to be positive for *C. sakazakii*. This evidence confirms the ubiquitous nature of this pathogenic organism. Moreover, contaminated utensils used for the preparation of infant feed, blenders and spoons, as well as prolonged storage of reconstituted infant milk formula (IMF) in bottle warmers has been linked to *C. sakazakii* infection of infants[2,41,42]. *Cronobacter sakazakii* has also been isolated from dairy products such as milk powders, cheese products and baby foods[43]. It has also been cultured from minced beef, sausage meat and vegetables[44]. Attempts have been made in the past to culture *C. Sakazakii* from other environmental and natural settings, including surface water, soil, mud, rotting wood, grain, bird droppings, domestic animals, cattle and cows' milk[45]. The organism was not detected in any of these environments. Although the mode of transmission is not always clear, contaminated IMF is a recognized source of C. *sakazakii*, which poses a serious risk of infection to newborn infants[6,46,47].

7. Infectious dose

The infectious dose for *Cronobacter* has not been determined, although Health Canada is working on a dose-response relationship[7]. The infectious dose will be influenced by the state of the bacteria, the immune system of the host and the environment in which the bacteria grew before infection. The proposed infectious dose value is 1000 cfu.100 g^{-1} although Pagotto *et al*[48] found that 10,000 cfu per mouse was the lowest count to be lethal in a suckling mouse assay. Nevertheless, it will take up to 9 days at 8 °C in reconstituted IFM for the pathogen to reach 1000 cfu.g-1, whereas it may take only 17.9 h at room temperature with a contamination level of 0.36 cfu.100 g-1.This model shows that it is unlikely that normal contamination levels would cause infection. The more likely possibility is temperature abuse and/or cross contamination from preparation utensils[13].

8. Pathogenicity and virulence factors of *Cronobacter sakazakii*

There has been little understanding of factors involved in the pathogenesis of *Cronobacter* at the molecular level. Pagotto *et al*[48] were the first to investigate putative virulence factors and the dose– response relationship in *Cronobacter* infection. The strains in this genus display differences in pathogenicity and may have different virulence factors[48,49,50]. *Cronobacter sakazakii, C. turicensis* and *C. malonaticus* are the only species which have been isolated from cases of neonatal meningitis. However, a strain belonging to *C. muytjensii* has been isolated from human bone marrow which would normally be sterile[10]. Little is known about the mechanism of infection or the different virulence factors of *Cronobacter*

spp. In mammalian tissue culture, the organism can attach to intestinal cells and survive internally in macrophages[51]. However, the specific bacterial adhesins and host cell receptors involved in these processes are unknown. Some strains of *C. sakazakii* produce capsular material and how this material contributes to macrophage evasion remains to be determined[39]. Furthermore, this capsule may also provide protection for the organism, facilitating its survival in desiccated environments. *E. sakazakii* can attach to plastics and silicon rubber surfaces and grow in a biofilm. Enteral feeding tubes and feeding-bottle teats can harbor the bacterium in large numbers[52]. Biofilm formation may also be a factor associated with altered susceptibility to antimicrobials[27,39].

One virulence factor of *Cronobacter* is the O-antigen. These polysaccharide side chains are variable and are responsible for serological diversity among bacteria. Two serotypes of the *rfb* locus which are implicated in the synthesis of the O-antigen were identified in *Cronobacter* strains. This has important implications for the virulence of *Cronobacter* since the O-antigen is a major surface antigen present in Gram-negative bacteria[53]. The O-polysaccharide produced by this strain is a linear unbranched polymer consisting of a repeating pentasaccharide unit. The structure of this O-polysaccharide differs in size according to sugar composition and complexity of the structure when compared to the O-polysaccharide structures of other *Cronobacter sakazakii* strains. These differences create diversity between serotypes[49] and may possibly reveal that *Cronobacter* is serologically heterogeneous with respect to the O-antigens[50]. Microarray analysis supports the observations that there are multiple O antigen serotypes, not only between *Cronobacter* species, but also within *C. sakazakii*.

Another virulence factor of the *Cronobacter* species is the production of proteolytic enzymes. Cell deformation, particularly "rounding" of cells, is a result of the action of various proteases[54] and *Cronobacter* strains have been found to cause this type of deformation of the tissue cells of mice[48]. In particular a zinc-containing metalloprotease were identified in *Cronobacter* cells which caused rounding of Chinese hamster ovary cells. This enzyme had collagenolytic (lysis of collagen) activity which may allow the pathogen to cross the blood-brain barrier or cause the extensive cell damage found in neonates with necrotizing enterocolitis. It was found that all of the strains tested possessed the *zpx* gene which codes for this proteolytic enzyme[55]. Additionally, *Cronobacter* strains have been found to produce an enterotoxin[48]. Purification and characterisation of this enterotoxin indicated its molecular mass as 66 KDa and that it is most active at pH 6. The enterotoxin proved to be highly stable as it was unaffected after incubation at 70 °C for 30 min and showed only a decrease in activity after 30 min incubation at 90 °C[56]. However, the importance of the enterotoxin is still unclear as the genes encoding the putative toxin and the protein itself remain unidentified[57].

9. Disease caused by *Cronobacter sakazakii*

Since the case of a *Cronobacter* infection in Tennessee was reported in 2002[58], the number of well documented cases worldwide has increased. However, the surveillance of the infections and number of incidences in different age groups are not sufficient to provide the exact number of infections attributed to this pathogen. At least 111 cases have been reported in infants and children of whom 26 were fatal[1,13,59,60,61]. Only a few of these cases are well described and most of them occurred sporadically, making epidemiological investigations impossible. In the case of IFM, the bacteria may exist in clumps rather than be spread out evenly throughout the product[62]. This may also lead to false negative results causing underestimation of contamination and the retail of contaminated products. The accurate determination of the occurrence of *Cronobacter* species is also greatly influenced by the low sensitivity and specificity of the detection methods for this genus. However, improvements have been made in the detection and identification methods that would aid in the accurate estimation of *Cronobacter* contamination[63]. These contributions will assist in the development of a reasonable risk assessment and consequent control of *Cronobacter.* While England, Wales, Scotland and Ireland hasthe most information about *Cronobacter* infections, countries such as Canada, Argentina and the Netherlands are making remarkable efforts to evaluate the risk and characteristics of *Cronobacter* spp.[7,64,65].

10. Characteristics of disease

The genus *Cronobacter* has been associated with sporadic infections and outbreaks. The first known cases of *Cronobacter* infections were two cases of meningitis in neonates[66]. These pathogens have been shown to cause a severe form of neonatal meningitis which is an acute inflammation of the membranes surrounding the brain and spinal cord[26]. *Cronobacter* strains have also been isolated from infants associated with necrotizing enterocolitis which is caused by

infection of the intestines. Other symptoms of infections include septicaemia[67], bloody diarrhoea[68] and brain abscess[69]. The mortality rate has been reported to vary from 10 % to 80 % with fatalities occurring just days after symptoms developed[25]. Generally *Cronobacter* affects the central nervous system[70] and survivors often suffer from severe neurological problems after the infections[9,69,71].

11. Mode of transmission

The sources of *C. sakazakii* and its vehicles of transmission are not always clear. Although the organism has been detected in multiple food sources, a strong association has been found only with PIF. Intrinsic and extrinsic contamination of PIF with *C. sakazakii* can occur. Intrinsic contamination results from the introduction of the organism to the PIF at some stage during the manufacturing process. In contrast, extrinsic contamination may result from the use of contaminated utensils, such as blenders and spoons; in the preparation of PIF[42].Several investigations into the presence of *C. sakazakii* in PIF have been performed. Muytjens *et al.*[71] (1988) examined 141 different powdered formulas from 35 countries and isolated *C. sakazakii* at levels ranging from 0.36 to 66 cfu per 100 g from 20 formula samples from 13 countries. *C. sakazakii* (8 cfu per 100 g) from PIF in association with an outbreak in Memphis, Tennessee[69]. *C. sakazakii* from 5 different lot numbers of unopened packages of PIF after an outbreak of neonatal meningitis in Iceland[46]. A Canadian survey that investigated the incidence of *C. sakazakii* in PIF isolated the organism from 8 of 120 cans from 5 different manufacturers[18]. An outbreak of *C. sakazakii* infection involving 12 infants who had necrotizing enterocolitis in 1998 in Belgium; *C. sakazakii* was isolated from liquid formula prepared from PIF[3]. In Belgium 2002, an infant died of *C. sakazakii*–associated meningitis after consuming a commercial PIF. The product was withdrawn after the detection of low levels of *C. sakazakii* in the implicated infant formula. In New Zealand in July 2004, a premature infant contracted *C. sakazakii* meningitis and died. The subsequent investigation found that 4 other babies in the neonatal intensive care unit were colonized with this organism, but none became unwell. The investigation attributed the source of the organism to PIF used in the nursery[72]. Most recently, another PIF was withdrawn after a possible link to 5 cases of presumed *C. sakazakii* infection in premature infants in France in 2004 that led to the death of 2 infants[73].

12. Antibiotic susceptibility

Members of the genus *Cronobacter* appear to differ considerably in terms of their susceptibility to various antibiotics. All the *Cronobacter* strains tested by Farmer *et al.*[10] were resistant to penicillin, whereas some strains were susceptible to chloramphenicol and ampicillin and only 13% of the strains were susceptible to cephalothin. In contrast *Cronobacter* strains were subsequently identified that were resistant to cephalothin and chloramphenicol, as well as ampicillin and tetracycline[18,74]. Recently the resistance of *Cronobacter* species to ampicillin, cephalothin and extended spectrum penicillin have been confirmed[9,20]. A recent clinical case has been reported in which multiple antibiotics including ampicillin, gentamicin and cefotaxamine were ineffective in the treatment of a *Cronobacter* infection[75]. Ampicillin in combination with chloramphenicol or gentamicin is also inefficient in the treatment of *Cronobacter* infections as the pathogen seems to be increasingly resistant to these antibiotics[9]. However, resistance to ampicillin has emerged owing to the acquisition of transposable elements and the production of β-lactamases[76,77]. *Cronobacter* species are known to be capable of inactivating broad spectrum penicillins and cephalosporins through the production of β -lactamase enzymes. This situation also appears to be increasing among isolates of *C. sakazakii.* Consequently, consideration should be given to the use of carbapenems or the newer cephalosporins in combination with a second agent, such as an aminoglycoside. The use of trimethoprim- sulfamethoxazole may also be useful[9].

13. Public health concern and food safety

Infants and young children are particularly vulnerable to food borne infections. Therefore, the microbiological safety of infant and follow-up formula is of utmost importance. Care givers in hospital neonatal units should be continuously alerted to the fact that PIF is not a sterile product and that, therefore, the use of hygienic measures during preparation and reconstitution are essential. PIF has been fed to millions of infants for years, and it constitutes the majority of infant formula used worldwide. This product is formulated to mimic the nutritional profile of human breast milk[58]. Because PIF is not a sterile product, it is an excellent medium to support bacterial growth. Bovine milk is an essential ingredient of PIF and a potential source of bacteria that are pathogenic to humans. On occasion, bacterial pathogens have

been cultured from PIF, including *Citrobacter, Enterobacter, Klebsiella, Staphylococcus, Streptococcus,* and *Yersinia* species. Should *E. sakazakii* multiply in PIF, it can result in infection[77]. A definitive link between the presence of *E. sakazakii i*n an unopened can of PIF and an outbreak of infection has been reported[58]. Cases of invasive *C. sakazakii* infection have recently been added to the list of notifiable diseases in New Zealand, after the death of an infant due to *E. sakazakii* meningitis in July 2004[72]. These actions highlight the importance of this opportunistic pathogen and the risk posed to vulnerable infants. The World Health Organization recommends that infants should be exclusively breast-fed for the first 6 months of life. Infants who are not breast-fed should be provided with a suitable breast milk substitute, formulated in accordance with Codex Alimentarius Commission standards. To reduce the risk of infection in infants fed PIF, recommendations have been made for the preparation and storage of PIF. A summary of the current guidelines to care givers in hospitals and the home is shown in table. Manufacturers of PIF is being encouraged to develop agreater range of commercially sterile alternative formula products for high-risk groups. In addition, formula manufacturers must implement strategies aimed at reducing the risks of product contamination. Controlling the initial populations of *C. sakazakii* during the production of PIF and avoiding post processing contamination, using suitable microbiological approaches, will have a positive effect. Data from surveys showed that *C. Sakazakii* can be cultured at various frequencies in samples of PIF, from the manufacturing facility, and from environmental sources[3, 40, 71]. However, the true frequency of contamination is unknown, making it difficult to quantify the level of risk to vulnerable groups. The role of the broader infant food chain and of dairy animals and their environment as sources of contamination has not been investigated. Standardized analytical approaches are necessary to ensure product safety. The European Food Safety Authority has recommended the introduction of a performance objective for PIF and followup formula that is aimed specifically at low levels of *Salmonella* and *C. sakazakii* (e.g., absence in 1, 10, or 100 kg)[44].

14. *Combined steps to reduce risks connected to C. sakazakii*

14.1 During production

- Monitor raw materials, specifically ingredients which do not require further thermal treatment before mixing
- Reduce level of *Enterobacteriaceae* in areas used for production in order to prevent subsequent contamination
- Increase frequency of inspections on food production environments and on the end product
- Identify the sources of possible contamination and take corrective measures
- Revise the instructions for milk preparation suggesting a higher water temperature, but not higher than 80 °C, for solubility (> 70 °C).A temperature too high would damage the nutritional characteristics of the product

14. 2 At home

- Use clean and disinfected containers
- Prepare only food enough for the meal avoiding the preparation of following meals; if necessary limit the number of meals prepared in advance to 1-2
- Avoid leaving unused reconstituted milk at room temperature
- Store in a refrigerator the reconstituted product
- Reduce as much as possible the lapse of time between the reconstitution of the formula and its use

14.3 In hospital/nurseries

- Follow practices of good hygiene in preparation are as
- Produce guidelines related to preparation, handling, preservation and control procedures for the product, and make them available to trained personnel
- Have access to a room reserved for preparation, which has an area for stocking the product and to which only authorized personnel have access
- If a dedicated room is not available, select an area reserved for this purpose
- Have access to utensils and equipment manufactured in order to be easily sanitized Sterilize all utensils used for preparation with thermal treatment (*e.g.*, washing in dish washers) or with autoclave
- Whenever possible use disposable utensils
- Employ qualified and specialized personnel(*i.e.*, dieticians)
- Store the reconstituted product in a refrigerator
- Reduce as much as possible the lapse of time between reconstitution of the product and its use
- Avoid leaving reconstituted milk at room temperature if unused
- Appropriately seal all containers with remaining milk, place them in the refrigerator, noting down an expiration

date
- Whenever possible, use milk in liquid form
- Enforce appropriate control measures which can assess potential hazards, identify critical control points (CCP), monitor non-conformities and necessary corrective actions, and register results

15. Conclusion

C. sakazakii is an emerging pathogen, often transmitted through powdered milk and responsible for a series of infections, some of which with potential fatal outcomes, in a particular segment of the population. Factors contributing to increase the risk of infection include: patient's susceptibility, level of contamination of food, tolerance to temperature, speed of growth, infectious dose and the virulence of the micro-organism. Reliable detection and accurate identification are paramount with respect to food and clinical environments. Although molecular methods of detection are generally faster compared with conventional microbiological phenotype-based methods, the requirement for specialized equipments and operator training deters some sectors from implementing these protocols.

Little is known about the *in vivo* virulence factors and pathogenicity of *Cronobacter* spp., which are crucial in the design of therapies to treat and control infections. A better understanding of the progression and pathogenesis of *Cronobacter* spp.-related diseases, particularly using in vitro cell-based assays combined with animal model studies is needed. Investigations into properties of *Cronobacter* that promote environmental persistence, pathogenicity and virulence factors, identification of environmental reservoirs and methods of elimination are active areas of research. Studies involving *C. sakazakii* have focused on methods to eliminate the coliform from powdered infant formula, to determine thermal resistance, environmental reservoirs, pathogenicity, antibiotic resistance, exopolysaccharide production and to develop rapid methods detection, enumeration and identification, subtyping and predictive modeling, but additional researches in these and other areas are needed. The ability of the pathogen to produce biofilms, coupled with its resistance to sanitizers and disinfectants when present in organic matrices, emphasizes the importance of properly cleaning and sanitizing food preparation areas and utensils and containers used to prepare and serve foods to neonates and others in hospital, daycare center, and home settings. Better understanding of the pathogenesis *C. sakazakii* - related diseases will help in the development of new modes of prevention for this emerging pathogen.

Regarding the ubiquitousness of *C. sakazakii* in inanimate (water, soil, plants) and animate environment (animals, man) it is not surprising, that *C. sakazakii* was detected in a wide spectrum of food and food products of animal and vegetable origin. Altogether, *C. sakazakii i*s not very frequent in food. Hygiene mismanagement due to incorrect temperature and time factors as well as due to the contact transmission of microorganisms via hands, insects, small vertebrates and equipment should be avoided during production, preparation and storage of food and drink.

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