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Review

Trending biocontrol strategies against *Cronobacter sakazakii*: A recent updated review

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ABSTRACT

Cronobacter sakazakii is an emerging foodborne pathogen, causing life-threatening infections in newborns and premature infants. *Cronobacter* spp. can survive under difficult processing conditions thereby contaminate the Powdered Infant Formula (PIF) during the manufacturing process. Infantile infections are associated with the consumption of contaminated PIF that was either contaminated intrinsically or extrinsically. This necessitates the development of sustainable strategies to manage the risk of *Cronobacter* infections. Natural methods of preservation holds promise as a viable alternative strategy to address the critical problem of emerging antimicrobial resistance and also to limit the negative effects of commonly used physico-chemical methods in food processing. The present study reviews the efficacies, potentials and developmental trends of biological antagonists and a combinatorial therapy to eliminate *C. sakazakii* using *in vitro* and *in vivo* methods. The mode of action of each biocontrol method has been discussed comprehensively. Most of these biocontrol agents interfere with the cell membrane integrity and its functions. However, none of the individual methods are able to eliminate the pathogen completely from the model food system i.e. reconstituted PIF. Each of the biological control strategies (agent) has its limitations in terms of their dose and method of application. A synergistic effect has been observed between the biological agent and physico-chemical treatments that may have the potential to ensure pathogen-free foods. Future research studies should evaluate the synergistic activities of these methods for their implication in infant foods as well as to understand the mechanisms of inactivation.

1. Introduction

World Health Organization (WHO) recommends breast milk as the best food for infants and recognized its promising health benefits (Boué, Guillou, Antignac, Le Bizec, & Membré, 2015). However, the infants and neonates which cannot be breastfed due to some unavoidable reasons are fed with reconstituted powdered infant formula (PIF) which is considered as an equivalent alternative to breast milk (Barron & Forsythe, 2007; Kent, Fitzgerald, Hill, Stanton, & Ross, 2015). In this regard, the quality and safety of PIF present a major challenge for food manufacturers and need to satisfy the International Microbiological criteria of 'Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (CAC/RCP 66–2008). Although PIF is a dehydrated product with water activity (a_w) of 0.25–0.35; being an unfavourable medium for the growth of most microorganisms, still there are several incidences where contaminated reconstituted PIF has been reported to be implicated in various infections of *Cronobacter sakazakii* in infants and neonates (Lepuschitz et al., 2019; Elkhawaga, Hetta, Osman, Hosni, & El-Mokhtar (2020)).

The recent taxonomic study reported seven different species under the Genus *Cronobacter* (formerly *Enterobacter sakazakii*) namely *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis, C. universalis*, and *C. condimenti* (Iversen et al., 2007, 2008; Joseph et al., 2012; Stephan et al., 2014). *C. sakazakii* being the most frequent clinical isolate of genus *Cronobacter* among all age groups comes under Group 1 (Forsythe, 2018). Along with *C. malonaticus* and *C. turicensis*, it is also known as the most pathogenic species reported to cause meningitis, sepsis, and necrotizing enterocolitis (NEC) in neonates and infants (Patrick, Mahon, Greene, Rounds, Cronquist, Wymore, & Bowen, 2014; Alsonosi et al., 2015; Finkelstein et al., 2019). The epidemiological data indicated that the infection caused by *C. sakazakii* has a fatality rate of 27% (Friedemann, 2009). Infants who survive,

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frequently suffer neurological sequelae such as quadriplegia, hydrocephalus and retarded neural development (Lai, 2001; Bowen & Braden, 2006; Bowen & Braden, 2008; Holý & Forsythe, 2014). Since the first two cases of neonatal infections caused by *Cronobacter* spp. (Urmenyi & Franklin, 1961) reported in 1961, to date there have been approximately 120–150 cases of *Cronobacter* infections in neonates and young children worldwide (Song, Teng, Chen, & Kim, 2018). The environmental reservoir of *C. sakazakii* is not evidently understood yet. However, various studies implicate reconstituted PIF as the major source of transmission (Postupa & Aldova, 1984; Muytjens, Roelofs-Willemse, & Jaspar, 1988; Simmons, Gelfand, Haas, Metts, & Ferguson, 1989; Biering et al., 1989; Muytjens & Kollée, 1990; van Acker et al., 2001; Weir, 2002; Parra-Flores et al., 2020). The studies on the prevalence of this pathogen in PIF always indicated a low level of contamination (< 1 cfu/100 g) (Iversen & Forsythe, 2003; Osaili & Forsythe, 2009); pointing towards occurrence of extrinsic contamination of PIF (FAO, 2007). Other than PIF, the presence of *C. sakazakii* have been also documented in a variety of abiotic sources including food processing units (Iversen & Forsythe, 2003; Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004; Singh, Goel, & Raghav, 2015), surfaces of utensils and equipment used for reconstitution of PIF (Kim, Ryu, & Beuchat, 2006; Noriega, Kotloff, Martin, & Schwalbe, 1990; Richards, Gurtler, & Beuchat, 2005) due to its ability to form biofilms (Iversen, Lane, & Forsythe, 2004; Lehner et al., 2005). These characteristics provide a physical obstruction and protection to cells against different control strategies such as thermal treatment, osmotic stress, starvation, detergents, antibiotics, disinfectants, and sanitizers which are routinely used in food processing units (Jung, Choi, & Lee, 2013; Kim, 2006; Park & Kang, 2014). Several researchers reported that *Cronobacter* spp. can tolerate high temperature (Nazarowec-White & Farber, 1997; Breeuwer, Lardeau, Peterz, & Joosten, 2003; Iversen et al., 2004; Edelson-Mammel & Buchanan, 2004; Asakura, Morita-Ishihara, Yamamoto, & Igimi, 2007; Chang, Chiang, & Chou, 2010) and desiccation (Barron & Forsythe, 2007), significantly.

The International Commission on Microbiological Specifications for Foods (ICMSF, 2002) also described *C. sakazakii* as a “severe hazard for restricted populations, life-threatening or substantial chronic sequelae of long duration” (Lehner, Tall, Fanning, & Srikumar, 2018). Due to severity of infections caused by *C. sakazakii*, it is essential to develop rigorous control measures to reduce the risks of contamination at each step during the production of reconstituted PIF following the established guidelines and recommendations of competent food safety authorities (Kent et al., 2015).

Nowadays, biological measures for the control of food-borne pathogens are becoming more attractive due to emerging antimicrobial resistance and consumer’s awareness of health concerns regarding chemical food additives and preservatives (Balciunas et al., 2013; Oliveira, Ferreira, Magalhães, & Teixeira, 2018). Use of bioprotective agents such as plant derived compounds, probiotics, bacteriophages and/or their metabolites exhibiting antagonistic effects are some of the approaches investigated against *C. sakazakii* thus far. Many reviews have appeared in recent years targeting the physico-chemical approaches for inactivation of *C. sakazakii* (Pina-Pérez, Rodrigo, & Martínez, 2016; Hu, Yu, & Xiao, 2018; Henry & Fouladkhah, 2019); however, the biological methods have not been discussed comprehensively so far. Therefore, the main objective of this review is to address the various biocontrol strategies used for the control of *C. sakazakii* and exploration of their impact on *C. sakazakii* growth in reconstituted PIF to ensure food safety.

2. Biological methods used to control *C. sakazakii*

The use of bioprotective microorganisms (probiotics, bacteriophages), plant derived herbal extracts and essential oils are some examples of biocontrol agents that have been developed against *C. sakazakii* (Fig. 1). The primary criteria for selection of a biocontrol agent include highly antagonistic activity against the pathogen, safe for human consumption and it should not alter organoleptic and nutritional quality of food products (Oliveira et al., 2018). The following sections described in depth the different biological agents tested against *C. sakazakii*.

2.1. Probiotics and prebiotics

Majority of research studies and meta-analyses have emphasized the role of probiotics belonging to Lactic acid bacteria (LAB) group as biocontrol agents to control *Cronobacter* as defined by use in *in vitro* and *in vivo* models (Table 1 and Table 2) (Dermyshi et al., 2017; Deshpande, Athalye-Jape, & Patole, 2018; Kumar, Bhat, Borthakur, Alrefai, & Dudeja, 2018). Highlighting the importance of probiotics or their spent media in controlling *C. sakazakii*, the first report from Collado, Isolauri, and Salminen (2008) determined the strain specific efficacy of probiotics via *in vitro* competitive exclusion assays. This study reported that the prior colonization of probiotic strains on human intestinal mucus layer before the colonization and infection by *C. sakazakii* reduced its ability to adhere to the mucus layer. Sharma and Prakash (2013) demonstrated that there was a 50% reduction in *C. sakazakii* viable cell counts when it was co-cultured with *Lactobacillus fermentum*, *L. casei* and *Pedicoccus acidilactici* during five minutes of contact time. Following studies used the spent culture medium of the probiotic strains to assess their anti-*Cronobacter* activities under *in vitro* or *in vivo* conditions. Awaisheh, Al-Nabulsi, Osaili, Ibrahim, and Holley (2013) reported the role of heat labile bacteriocins, pH-neutralized cell-free supernatants (CFS) of *L. acidophilus* or *L. casei* with anti-*Cronobacter* activity. Although, the bacteriocins are Generally Recognised as Safe (GRAS), the heat labile nature of these proteins would not be applicable for utilization in the production of dehydrated PIF. Charchoghlyan et al. (2016) reported the role of organic acids from culture filtrate of *L. acidophilus* n.v. Er2 317/402 strain Narine, (an isolate from new borne baby feces) in inhibition of *C. sakazakii*. It is thought that un-dissociated form of acid molecules rapidly enters the cells, which causes an increased acidity of cytoplasm resulting in the disintegration of cytoplasmic membrane.

The above mentioned reports used pure cultures of probiotics or their supernatants. However, a mixed culture consortium has been reported to provide better inhibition against the pathogens (Ghosh, Chowdhury, & Bhattacharya, 2016). In this context, anti-*Cronobacter* activity of Kefir (fermented dairy product containing an undefined population of microorganism) supernatant has been reported. The Kefir supernatant was reported to completely inhibit the growth of *C. sakazakii* after one hour of exposure at a concentration of 30–50% in the reconstituted milk formula (Kim et al., 2015). Similarly, another report from Chang et al. (2018) reported the comparative anti-*Cronobacter* activity of Kefir and yogurt containing multiple strains of LAB and *Bifidobacteria*. Among the tested products, the Kefir types (mild with pH of 4.5 and strong with pH of 3.6) were shown to be more potent than yogurt types (containing LAB or LAB-Bifido combination) against *C. sakazakii* and inhibited the pathogens within 1–5 days of storage at 4 °C. Recently, Kim et al. (2018) reported the anti-*Cronobacter* activities of pure cultures of LAB, *L. kefiri* DH5 and *L. kefiranofacins* DH101 isolated from Kefir grains. The inhibition in these studies may be attributed to several bioactive compounds such as microorganisms present in product (live or dead) (Chon et al., 2013), the breakdown products from food matrix (peptides) and the metabolites produced by microorganisms during fermentation such as organic acids, polysaccharides, bacteriocins, etc. (Garrote, Abraham, & De Antoni, 2000; Guzel-Seydim, Kok-Tas, & Greene, 2011).

The results obtained from *in vitro* studies towards the control or inhibition of *C. sakazakii* need to be further validated using other *in vitro* models such as Caco-2 cell line and rabbit, mice and nematodes as an *in vivo* model. The studies using *in vivo* models provide an insight into the mechanisms along with target functions of probiotics (Gibson et al.,

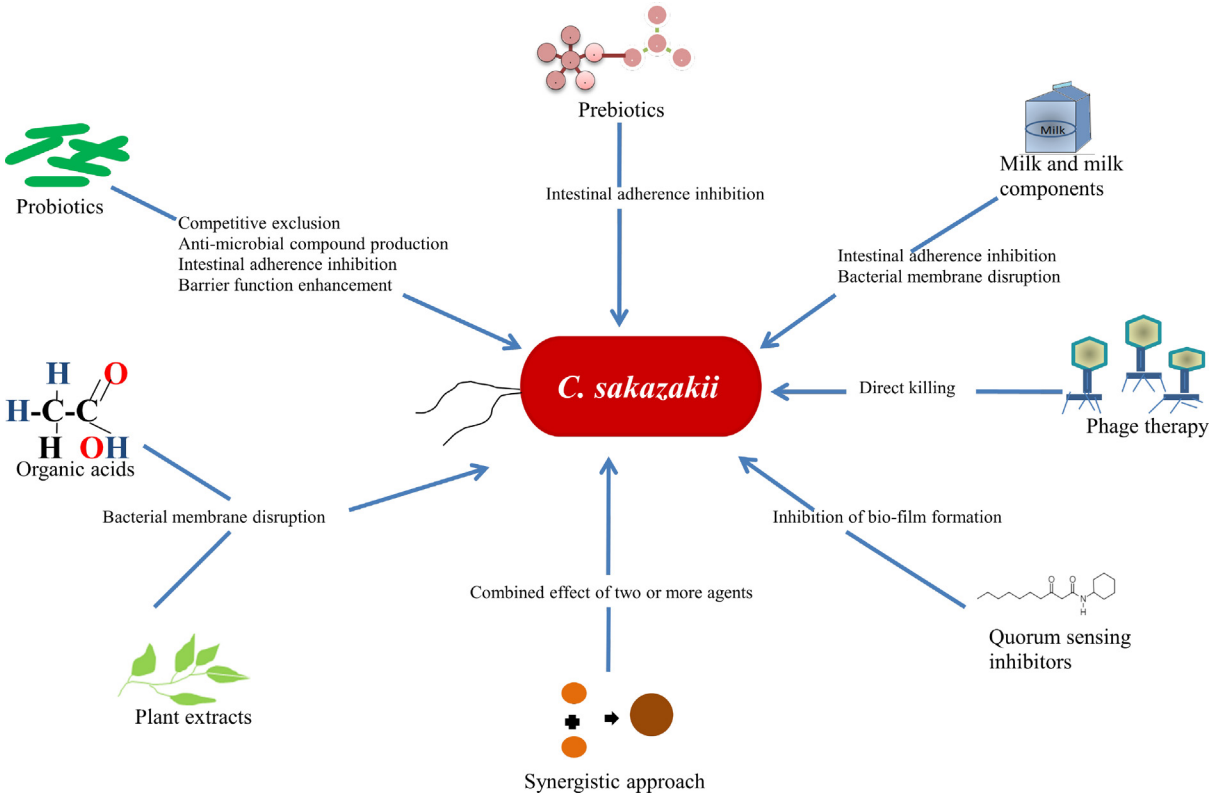

Fig. 1. Mode of action of different biocontrol agents against *Cronobacter sakazakii*.

Table 1
In vitro studies using probiotic lactic cultures and their metabolites against different strains of *C. sakazakii*.

Probiotic culture	Protection mechanism	Target Strain	Reference
Streptococcus thermophillus NCC2496 *Lactobacillus rhamanosus* NCC4007 *Lactobacillus paracasei* NCC2461 *Bifidobacterium longum* NCC3001 *Bifidobacterium lactis* NCC2818 (10^8 cells/ml)	Competitive exclusion along with inhibition and displacement	*C. sakazakii* (10^8 cells/ml)	Collado et al., 2008
Lactobacillus acidophilus *Lactobacillus casei*	Heat labile bacteriocin production	*C. sakazakii* isolates from infant formula	Awaisheh et al., 2013
Lactobacillus fermentum *Lactobacillus lactis* *Lactobacillus casei* *Pediococcus acidilacti* (10^3 CFU/ml)	Not specified	*C. sakazakii* MTCC 2958 and 5 *C. sakazakii* isolates (10^3 CFU/ml)	Sharma & Prakash, 2013
Kefir	Not specified	20 clinical and food isolates of *C. sakazakii* and *C. sakazakii* ATCC 29544	Kim et al., 2015
Lactobacillus acidophilus n.v. Er.2317/402 strain Narine	Cytoplasmic acidification caused by narine crude cell free extract	*C. sakazakii* ATCC 29544	Charchoghlyan et al., 2016
Multiple LAB yogurt Multi-LAB-BIF yogurt Mild kefir (pH 4.5) Strong kefir (pH 3.6)	Not specified	*C. sakazakii* ATCC 29544 *Salmonella enteritidis* 108 (~6 log CFU/ml)	Chang et al., 2018
Lactobacillus kefiri DH5 *Kefiranofacience* DH101 *Bifidobacterium longum* (positive control)	Antagonistic activity through organic acid, caused permeabilization of cellular membrane	*C. sakazakii* ATCC 29544	Kim et al., 2018
Various lactic cutlures	Not specified	*C. sakazakii*	Singh et al., 2020

2011; van Loveren, Sanz, & Salminen, 2012) (Table 2). Similar to the *in vitro* study conducted by Collado et al. (2008) on competitive exclusion of *C. sakazakii* by probiotics, Zhong, Lin, Long, Wu, and Fan (2015) have reported the competitive inhibition of *C. sakazakii* by *L. rhamnosus* GG via competing for adhesion to Caco-2 cells as well as on intestinal layers in neonatal rats. The dose dependent inhibition by the probiotic strain was reported via inhibiting the entry of the pathogen across the intestinal barrier preventing meningitis in the neonatal rat model Weng, Ganguli, Zhu, Shi, and Walker (2014) reported protective effects of *Bifidobacterium infantis* against *C. sakazakii* induced inflammation in neonatal mice model via maintaining the viability of ileum epithelium cells (anti-apoptotic effect) using probiotic conditioned medium (PCM). The authors also suggested that nuclear translocation of NF-κB p65was involved in and play a role as a key mechanism (Weng et al., 2014).

Table 2
In vivo studies using probiotic lactic cultures against infection of *C. sakazakii*.

Probiotic culture	*In vivo* model	Protection mechanism	Targeted strain	Reference
Bifidobacteria infantis (ATCC 15697) derived conditioned medium	C57BL/6 Mice	Anti-apoptotic effect and inhibition of *C. sakazakii* proliferation	*C. muytjensii* ATCC 51329	Weng et al., 2014
Lactobacillus rhamnosus ATCC 53103, *Lactobacillus plantarum* ATCC 10241	Rat pups	Not specified	*C. sakazakii* ATCC BAA-894	Blackwood et al., 2017
Lactococcus lactis	White rabbit pups	Not specified	*C. sakazakii*	Gurien et al., 2018

Probiotic cultures, *L. rhamanosus* and *L. plantarum* have also been reported to strengthen the tight junction integrity and intestinal barrier function in NEC induced Caco-2 cell and rat models (Blackwood et al., 2017). A similar study by Gurien, Stallings-Archer, and Smith (2018) also reported that administration of *Lactococcus lactis* in NEC induced preterm rabbit model reduced the frequency of NEC development by 51% with an increased survival rate of pups by 26% as compared to rabbits receiving *Cronobacter* contaminated feed alone. With specific regard to biofilm forming ability of *C. sakazakii*, Jamwal, Sharma, Chauhan, Bansal, and Goel (2018) reported the role of organic acids or bacterial metabolites in cell free supernatant (CFS) of commercial strains for their anti-biofilm activity. Nevertheless, the studies on efficacy of probiotics in limiting *in vitro* and *in vivo* infection of *C. sakazakii* are convincing which needs further validation for their use in food processing systems. In spite of the evidence for their efficacy in neonatal care, not all probiotics have similar effects in a specific disease state and caution must be taken into account before their prophylactic usage to ensure the safety and specificity in their action (Embleton, Zalewski, & Berrington, 2016; Yazdankhah et al., 2008; Zbinden, Zbinden, Berger, & Arlettaz, 2015).

In recent years, prebiotics were delineated as food ingredients offering several promising beneficial effects by influencing the bacterial adhesion to the gastro-intestinal tract (GIT) (Hickey, 2012; Kent et al., 2015; Lane, Mehra, Carrington, & Hickey, 2010). Additionally, Quintero et al. (2011) evoked that prebiotics may have an inhibitory effect on *Cronobacter* intestinal adherence, and consequently may provide clinical protection from this pathogen. Prebiotics, in particular polydextrose (PDX) and galacto-oligosaccharides (GOS), alone and/or in combination have been reported to exert an anti-adhesive effect on *C. sakazakii* and directly decrease the adherence of this pathogen to Hep-2 cell lines during the establishment of initial infection stage. The GOS at a level of 16 mg/mL significantly decreased the adherence of *C. sakazakii* to epithelial cell lining. Furthermore, the blend of GOS-PDX (8 mg/mL) resulted in 48% decrease in adherence of the pathogen. These results are interesting although the concentration of both prebiotics analyzed was higher than the concentrations used in PIF. Moreover, *in vivo* investigations further suggest and confirm the mode of action of prebiotic substrates. Milk glycans are another potential prebiotic sources that have an ability to interfere with the attachment of pathogenic bacteria to gut epithelial cells mediated via the structural similarity of milk oligosaccharides (OS) to the carbohydrate ligands on the host cell surface that serve as receptor sites for bacterial adherence (Clare, Catignani, & Swaisgood, 2003; O'riordan, Kane, Joshi, & Hickey, 2014).

2.2. Bioactive peptides/milk and milk product's derived components

Mammalian milk/Milk-derived from different sources besides their nutritive role also possess several other health promoting properties including antimicrobial activity through a variety of components including glycolipids, glycoproteins (such as lactoferrin, mucin, immunoglobulins, etc.), lactoperoxidase system and oligosaccharides (Murad, 2014; Panwar, 2014). Several studies have shown the promising antimicrobial activity of these compounds against *C. sakazakii* (Table 3). Bovine colostrum is reported to contain many bioactive components such as insulin like growth factor I and II (IGF-I and IGF-II), lysozyme, lactoperoxidase, lactoferrin and immunoglobulins (Tripathi & Vashishtha, 2006). Adherence inhibition of *C. sakazakii* by bovine colostrum fractions, their ultra-filtered (UF; < 10,000 Da), nano-filtered (NF; < 1000 Da) filtrate and nano-filtered retentate (1000–10,000 Da) was assessed in HEp-2 cells (Maldonado-Gomez, Lee, Barile, Lu, & Hutkins, 2015). The study reported 99% adherence inhibition of *C. sakazakii* by bovine colostrum fractions at a minimum concentration of 10–20 mg/mL of UF and 40 mg/mL of NF retentate. The NF retentate did not show any significant inhibitory activity against *C. sakazakii*. Apart from bovine milk, raw and pasteurized camel milk

Table 3
In vitro studies on Milk or milk derived fermentate for inhibition *C. sakazakii.*

Milk and milk compound	Protection mechanism	Targeted strain	Reference
Lactoperoxidase system	Not specified	Food, environmental and clinical isolates of *C. sakazakii*	Gurtler & Beuchat, 2007
Bovine colostrums fractions	Acidic oligosaccharides as an anti-adhesive component	*C. sakazakii* 4603	Maldonado-Gomez et al., 2015
Raw and pasteurized camel milk	Not specified	*C. sakazakii* DSM 4485 *C. sakazakii* isolated from PIF (~4 log_{10}CFU)	Abusheliabi et al., 2017
Commercial butter milk, butter serum, skim milk and raw butter milk	Not specified	*C. sakazakii* CECT 858 (~10^7CFU/ml)	Ripollés et al., 2017
Lactobacillus acidophilus DPC6026 derived casein fermentate	Through anti-microbial peptides (caseicin A and caseicin B) obtained from caseinate fermentation	*E. sakazakii* NCTC 8155 *C. sakazakii* ATCC 12868	Hayes et al., 2009
Lactoferrin	Cell membrane disruption	Four *C. sakazakii* food isolates	Al-Nabulsi et al., 2009
Native and iron saturated bovine lactoferrin	Iron sequestration	*C. sakazakii* CECT 858 (~10^4CFU/ml)	Harouna et al., 2015
Lactoferrin	Not specified	*C. sakazakii* 4603	Quintero-Villegas et al., 2014

were reported to reduce the growth of *C. sakazakii* by 0.6% and 4.6% respectively at 25 °C while by 5.1% and 10.5% at 37 °C (Abusheliabi et al., 2017). The higher levels of heat stable antimicrobial components were suggested to be responsible for this anti-*Cronobacter* activity which could serve as a promising noble candidate for PIF supplementation taking into consideration of guidelines issued by food supplement regulating agencies. Ripollés et al. (2017) reported the anti-adhesive potential of commercial buttermilk at 20 mg/mL among skimmed milk, butter serum and raw buttermilk tested at different concentrations (2, 10, 20 mg/mL) using an ELISA based assay in Caco-2 cells against *C. sakazakii*. It was reported that the heat treatment which is applied to production of these products during processing might lead to specific unfolding of protein which might interact with the bacterial surface (Raikos, 2010), forming antimicrobial fat globules (Guri, Griffiths, Khursigara, & Corredig, 2012) and generation of Maillard reaction metabolites leading to damage of bacterial cell membrane (Rurián-Henares & Morales, 2008).

Besides these milk components, activation of lactoperoxidase system (LPOS) in milk results in production of two antimicrobial intermediates, hypothiocyanite ion ($OSCN^-$) and hypothiocynous acid ($HOSCN^-$) which possess inherent antimicrobial activity. These intermediates exert an antimicrobial effect by oxidising sulfhydryl groups on enzymes and proteins associated with the bacterial cytoplasmic membrane (Kussendrager & Van Hooijdonk, 2000). Gurtler and Beuchat (2007) revealed the antimicrobial activity of the lactoperoxidase system (LPOS) against *Cronobacter* in reconstituted PIF stored at 21, 30 and 37 °C. The enriched formula did not show any growth of *C. sakazakii* (less than 1 CFU/227 mL) when treated with 10–30 μg/mL or 30 μg/mL LPOS and stored for 24 h at 30 and 37 °C, respectively. The study also suggested that LPOS intervention either during manufacturing or during reconstitution of PIF is unaffected by heat treatment.

Milk proteins are regarded as one of the inexpensive and promising sources of bioactive peptides and hydrolysates (Kent et al., 2015; Rani, Pooja, & Pal, 2017). These peptides can act either via disrupting bacterial cell membrane or via denaturing nucleic acids and intracellular proteins (Kragol et al., 2001). Hayes et al. (2009) assessed the anti-*Cronobacter* activity of sodium caseinate fermented with proteolytic strain, *L. acidophilus* DPC 6026. The fermentate possessed higher quantities of bioactive peptides (IKHQGLPQE-Caseicin A, VLNENLLR-Caseicin B) which inhibited *C. sakazakii* growth in reconstituted PIF. The dose dependent antibacterial activity of pH neutralized fermentate completely inhibited *C. sakazakii* at 3.33% w/v of fermentate over a time of 60 min. Lactoferrin (LF), another iron binding protein is one of the well-known antimicrobial peptide found in the various types of milk. Quintero-Villegas, Wittke, and Hutkins (2014) reported that native lactoferrin (nLF) (10 mg/mL) alone and in combination with other prebiotics such as galacto-oligosaccharides (GOS):Polydextrose (1:1) reduced the adherence potential of *C. sakazakii* to Hep-2 cells. The activity was suggested to be mediated either by binding of lipid A portion of LPS of Gram negative bacteria or disruption of type III secretory systems (Atef Yekta et al., 2010). Another study on the native and iron saturated bovine lactoferrin (bLF) demonstrated that only native bLF is able to inhibit *C. sakazakii* in milk or whey based media (Harouna et al., 2015). The study also reported that heat stable native bLF mediated the inhibition of *C. sakazakii* via iron sequestration (Harouna et al., 2015). The same group has also reported that hydrolysate of bLF in whey when combined with undigested bLF, resulted in complete inactivation of the pathogen after 8 h of incubation at 37 °C (Harouna et al., 2020). Recently, a recombinant protein, Funme peptide was reported to possess anti-*Cronobacter* activity in reconstituted PIF with MIC of 125.0 μg/ml and has an ability to inhibit biofilm formation. The mechanism was suggested due to increased permeability and the release of cytoplasmic β-galactosidase of the pathogen (Chen et al., 2019). However, the concentration of these antimicrobial peptides (AMP) in milk is low and production of such AMPs at commercial scale has been hindered due to lack of large scale reproducible technologies. Therefore, there is an urgent need to develop such commercially viable processes.

2.3. Phage therapy

The emergence of antimicrobial drug resistance in foodborne pathogens has led to the application of bacteriophages (viruses that infect bacteria) for the control of pathogens as an alternate at each stage of farm to fork continuum (de Melo, Levesque, & Moineau, 2018). Since the approval of first phage based product (LiestShied™) to overcome *Listeria monocytogenes* contamination in meat and poultry in 2006, a number of phage products have been approved by FDA (Bren, 2007). Some reports are there describing the potential of phages as biocontrol agents to control *C. sakazakii* contamination in food processing units and in after processing practices (Table 4). A study by Kim, Klumpp, and Loessner (2007) achieved complete inhibition of *Cronobacter* spp. in reconstituted PIF at high doses (10^9 pfu/mL) of *Cronobacter* specific bacteriophages. An *in vivo* study employing *Cronobacter* spp. specific phage to prevent renal colonization by *Cronobacter* spp. in a mice model of urinary tract infection showed that phage therapy could reduce colonization of pathogen up to 70% (Tóthová et al., 2011). The elevated expression of malondialdehyde, pro-inflammatory cytokines, monocyte chemoattractant protein-1 and tumor necrosis factor-alpha (TNF-α) was reduced in mice model. The results of these experiments suggest that phage therapy could also modulate the host's immune response. Another study was conducted to determine *in vivo* efficiency of phage therapy by using *Galleria mellonella* (greater wax moth) larvae as a whole animal model, whereby the phage administration at a

Table 4
In vitro and *in vivo* studies on phage therapy for control of *C.sakazakii*.

Bacteriophage	Targeted strain	Reference
C. sakazakii specific pages (10^9 PFU/ml)	*C. sakazakii* ATCC 29544 and five food isolate	Kim et al., 2007
Cocktail of five phages (10^4–10^8 PFU/ml)	40 *Cronobacter* spp. strains (10^2–10^6 CFU/ml)	Zuber et al., 2008
vB_CsaM_GAP161 phage (2×10^9 PFU/ml)	*Galleria mellonella* larvae infected @ of 1.0×10^6 CFU/ml with *C. sakazakii*	Abbasifar et al., 2014 (*in vivo* study)
Bacteriophage CR5 (Accession No. JX094500)	*C. sakazakii* ATCC 29544 (10^2 cfu/ml)	Lee et al., 2016
Cocktail of three phages leE, leB, leN (Accession No. KX443552, KX431559B, KX431560 Respectively)	*C. sakazakii* ATCC BAA 894, *C. sakazakii* ATCC BAA LUX, *C. sakazakii* DPC 6258, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC DPC 8155	Endersen et al., 2017

multiplicity of infection (MOI) of 8 reduced mortality rate of *C. sakazakii* infected larvae to about 16% (Abbasifar et al., 2014). Lee et al. (2016) isolated and characterized lytic *C. sakazakii*-infecting bacteriophage CR5 having bacterial flagella as a possible receptor for host-phage interaction. Complete growth inhibition of *C. sakazakii* was reported within 2 h in PIF when phage CR5 was added at MOI of 10^4 or 10^5. Phages to be used as biocontrol agent in food must be exclusively lytic (non-temperate phages) and free of genes encoding for bacterial virulence factors (Endersen et al., 2014). However, the cost effective scale-up strategy for phages and identification of possible risks associated with phage application needs to be considered before their application in foods.

The role of bacteriophage endolysins as a biocontrol agent has also been reported. For this, endolysin LysSs1 from *Cronobacter* phage vB_CsaP_Ss1 was suggested as a novel candidate for biocontrol of Gram negative pathogens (Endersen et al., 2015). Although the activity of phages is specific for strains, however to increase the range of efficacy of phages, a cocktail of phages has been reported for broad host range. Zuber et al. (2008) reported a sterilizing effect of a cocktail in artificially spiked PIF in 35 out of 40 *Cronobacter* spp. test strain. The cocktail comprised of five phages at a concentration of 10^9 pfu/mL. Endersen et al. (2017) has reported 73% inhibition of *Cronobacter* strains using a cocktail of three *Cronobacter* phages leB, leE, leN in different brands of PIF. The phage cocktail (3×10^8 pfu/mL) was reported to reduce the cell number below the detection limit (< 10 CFU/mL). In addition to this, phage cocktails effectively interrupted the establishment of biofilms. As far as bacteriophages are concerned, their specificity makes them a good candidate as anti-*Cronobacter* agents since phage particles do not invade the eukaryotic host cells. However, they may stimulate humoral immune response. Therefore, long term studies dealing with safety aspects of phages are nevertheless needed for its application in food safety systems. Moreover, results obtained from *Cronobacter* bacteriophage studies inferred that high phage concentration is needed to achieve required product safety which in turn may raise infant safety questions with respect to phages. Along with this, there will be a need for development of fiscally sound commercial technology to make large enough quantities of phage to make the use of these biocontrol agents feasible.

2.4. Plant derived natural compounds

Many naturally occurring phytochemicals found in plants, herbs, and spices have been reported to possess anti-*Cronobacter* activity (Table 5). These phytochemicals possess negligible toxicity to human hosts, lower environmental impact and high public acceptance (Arya, Gupta, Verma, Pal, & Saxena, 2019; Oliveira et al., 2018; Pisoschi et al., 2018; Rani, Pooja, & Pal, 2018). Among the tested polyphenols, Red muscadine (*Vitis rotundiolia* Michx.) juice, a rich source of polyphenols and organic acids was shown to possess strong bactericidal activity against *C. sakazakii* and reducing its population by 6 log CFU/mL (initial population 6.5 log CFU/mL) within 2 h at 37 °C (Kim, Weng, Silva, Jung, & Marshall, 2010). The study suggested that various organic acids particularly tannic acid (1.7 mg/mL) played an important role in the reduction of *C. sakazakii* population. The chelation of iron from the medium was suggested as a mechanism of action of tannic acid which is quite different from mechanisms of other organic acids. The study from Joshi, Howell, and D'Souza (2014) reported the efficacy of blueberry proanthocyanidins (PAC) and commercial blueberry juice (BJ) against two strains of *C. sakazakii*, whereby the pathogen population was reduced to undetectable levels after 1 h at 37 °C. Furthermore, they have found that blueberry juice (BJ) and blueberry PAC have a bacteriostatic effect against *C. sakazakii* strains. The inhibition was suggested due to bleb formation and cell membrane damage due to pore formation (as indicated by scanning electron microscopic studies), disturbing cell membrane fluidity, changing fatty acid profiles, and destroying cellular metabolism. Tea polyphenols (TP), a richer source of catechins, flavanoids, phenolic acids and anthocyanins were also tested against *C. sakazakii* strains and results of the study indicated that the population of *C. sakazakii* strains were reduced up to 7 log CFU/mL within 1 h. Moreover, TP after acidification with HCl (pH 3.5) in rehydrated PIF showed a stronger antibacterial activity (Li et al., 2016). The study indicated that TP had an effective irreversible bactericidal effect against *C. sakazakii* mainly due to the disruption of outer and inner membrane of the pathogen. Recently, olive oil polyphenol extract (OOPE) (a natural biopreservative and biocide) was shown to have anti-*Cronobacter* activity (Fei et al., 2018). OOPE was found to be more potent than blueberry proanthocyanidins and tea polyphenols with minimum inhibitory concentration (MIC) values in a range of 0.625 to 1.25 mg/mL. The observed effect was suggested due to the presence of a hydroxyl group on phenolics (Ultee, Bennik, & Moezelaar, 2002).

The application of Essential Oils (EO's) in food systems as natural inhibitors or bio-preservatives mainly due to their antioxidant and antimicrobial properties; is an exciting and growing area of research (Calo, Crandall, O'Bryan, & Ricke, 2015; Fernández-López & Viuda-Martos, 2018; Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017). A series of five natural organic compounds; carvacrol, thymol, eugenol, diacetyl, cinnamic acid alone and in combination with nisin was investigated for controlling the growth of *C. sakazakii*. The highest inhibitory effect was recorded for carvacrol and thymol with a MIC value of 1.25 mmol/L. No significant enhancement in inhibition was observed for tested organic compounds in combination with nisin except for diacetyl (Lee & Jin, 2008). Amalaradjou, Hoagland, and Venkitanarayanan (2009) evaluated trans-cinnamaldehyde (extract obtained from cinnamon) for its anti-*Cronobacter* potential at different storage temperatures (37, 23, 8 and 4 °C). A higher growth inhibitory activity of TC (0.5%) was observed (up to undetectable level within 4 h of incubation) at higher temperature of 37 and 23 °C compared to storage at 8 and 4 °C. Further, the same authors appraised the potential use of TC in reducing the environmental stress tolerance of *C. sakazakii*. They reported that TC at its sub-inhibitory concentration (SIC) of 750 μM down-regulated *C. sakazakii* genes decisive for stress tolerance and survival, including *rpoS*, chaperonins, outer membrane porins, *phoP/Q*, and osmolyte transporter genes whereby *rpo*S was the only dominant gene for survival of *C. sakazakii* in all stress conditions.

Yemiş, Pagotto, Bach, and Delaquis (2011) demonstrated anti-*Cronobacter* activity of vanillin and its derivatives such as ethyl vanillin and vanillic acid with MICs ranging from 2 to 8 mg/mL. Further, the reduction in viable count of desiccated and non-desiccated cells of *C.*

Table 5
Antimicrobial activities of plant derived compounds against *C. sakazakii*.

Plant extract	Protection mechanism	Targeted strain	Reference
Carvacrol, thymol, eugenol, cinnamic acid and diacetyl	Not specified	*C. sakazakii* ATCC 29004, *C. sakazakii* ATCC12868 (10^6 CFU/ml)	Lee & Jin, 2008
Trans-cinnamaldehyde (TC)	Down regulation of responsible genes for stress tolerance and virulence associated genes	*C. sakazakii* ATCC 51329 and , *C. sakazakii* 4581, *C. sakazakii* 415 (meningitis isolate)	Amalaradjou et al., 2009, Amalaradjou & Venkitanarayanan, 2011
Red muscadine juice	Not specified	Two *C. sakazakii* strains	Kim et al., 2010
Vanillin, ethyl vanillin and vanillic acid	Cell membrane disintegration	Seven *Cronobacter* strains	Yemiş et al., 2011
Blueberry proanthocyanidins and blueberry juice	Cell membrane deformation	*C. sakazakii* ATCC 29544, *C. sakazakii*ATCC 29004	Joshi et al., 2014
Thymol, Carvacrol, Thymoquinone, Citral, Trans-Cinnamaldehyde, Thujone, p-Cymene, Eugenol, Linalool, Camphor, 1,8-Cineole, Limonene, γ-Terpinene, Bornyl acetate, Camphene, A-pinene, Curcumin, β-pinene, β- Caryophyllene, Cinnamon, Oregano, Lemongrass, Clove, Laurel, Propolis	Not specified	*C. sakazakii* ATCC 29544	Fraňková et al., 2014
Vanillin	Not specified	Cocktail of four *C. sakazakii* food isolates	Obaidat et al., 2015
Essential oils of cinnamon, eucalyptol, anise, fenugreek, fir, fennel, pooh, qysoom and rosemary	Not specified	*C. sakazakii* strains isolated from powdered infant milk formula	Al-Nabulsi et al., 2015
Methanolic extract of *Piper nigrum, Trigonella foenum-graecum, Coriandrum sativum, Cuminum cyminum, Syzygium aromaticum, Myristica fragrans, Zingiber officinale, Allium sativum and Cinnamomum verum*	Inhibition of acyl homoserine lactones (AHL) based quorum sensing	*C. sakazakii* ATCC 12868, *C. sakazakii* ATCC E604 and three environmental isolated *C. sakazakii* strains	Singh et al., 2016
Tea polyphenol	Cytoplasmic leakage	Four *C. sakazakii* strains isolated from powdered infant formula	Li et al., 2016
Olive oil polyphenol extract	Plasma membrane disruption, cytoplasmic leakage, protein and ATP conc. reduction	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC BAA-894, *C. sakazakii* ATCC12868, and four other isolates	Fei et al., 2018
Thymoquinone	Reduced transcription of sixteen virulence related genes (*fliD, flhD, flgJ, ompA, ompX, uvrY, motA, motB, sod, bcsA,bcsG, lpx, wzx, luxR, gale and kpsT*)	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC BAA-894, *C. sakazakii* ATCC12868, and six other isolates	Shi et al., 2017
Citrus sinensis, Cinnamomum zeylanicum, Piper chaba, Terminalia arjuna, Terminalia chebula, Mangifera indica, Allium sativum, Zingiber officinale, Azadirachta indica, Ocimumsantum, Syzygium aromaticum, Terminalia bellirica and *Emblica officinalis*	Not specified	*C. sakazakii* MTCC 2957, and one food isolate	Sharma & Prakash, 2013

sakazakii in reconstituted PIF by vanillin (12 mg/g) was also reported (Obaidat et al., 2015). Various studies have also reported that TC and vanillin derivatives mainly target the cytoplasmic membrane integrity via the accumulation of hydrophobic phenolic groups (hydroxyl group) in the lipid bilayer which disrupts the lipid-protein interactions, change the membrane structure, function, and permeability, accelerating the leakage of cell contents, interrupting the proton-motive force and electron influx, and ultimately destroying the cell integrity (Char, Guerrero, & Alzamora, 2010).

Fraňková et al. (2014) reported 12 EOs with MIC < 1 mg/mL; thymol, carvacrol and trans-cinnamaldehyde being the most effective with MIC value 0.1–0.3 mg/mL in the liquid phase. Among the EO's, cinnamon and oregano were the most effective among five tested EO's. A combination of fir and cinnamon oils was shown to reduce the *C. sakazakii* population up to undetectable level within 3 h of contact in reconstituted PIF (Al-Nabulsi et al., 2015). In another study on EO's, citral, a flavoring compound in citrus oils was reported to possess MICs of 0.27 to 0.54 mg/mL against *C. sakazakii* strains (Shi et al., 2016). An overall inference from the study on essential oils indicated that citral is the most effective antimicrobial agent against *C. sakazakii* at lower dose (Shi et al., 2016) targeting cytoplasmic membrane integrity. Sharma and Prakash (2013) provided insights into the susceptibility of *C. sakazakii* to many plant derived products. The authors reported that among 13 plant extracts, the alcoholic extract of *Terminalia chebula* was found to be most effective against *C. sakazakii* with a lower MIC of 0.2 μg/mL. The use of EO's in food is limited as they are associated with off-flavor and odors which may affect the overall acceptability of the food products. Moreover, many studies showed that a higher concentration of EO's is required in food commodities to achieve the same effect as observed in culture media (Bajpai, Baek, & Kang, 2012; Oliveira et al., 2018).

Anti-*Cronobacter* activity of organosulfur compounds was assessed using two stable garlic-derived organosulfur compounds (diallyl sulphide and Z-ajoene) with a cocktail of five *C. sakazakii* strains (Feng et al., 2014). Ajoene had a higher activity than diallyl-sulfide whereby ajoene at a concentration of 0.77 mM reduced *C. sakazakii* count to undetectable within 8 h. A dose-dependent concentration effect was observed which was indirectly proportional to the time of contact. The high throughput whole-transcriptome sequencing (RNA-sequencing) and vibrational spectroscopic analysis indicated that ajoene inhibited the expression of motility related genes in particular while diallyl-sulfide resulted in up regulation of cell wall synthesis genes. Both the compounds could freely penetrate the cell membranes and combine with a thiol-containing enzyme and/or protein, altering their structures and ultimately caused inactivation of the pathogens (Feng et al., 2014). Anti-*Cronobacter* effect of Coenzyme Q_0 (CoQ0, 2, 3-dimethoxy-5methyl-1,4-benzoquinone), found in *Antrodia cinnamomea*, has been reported. A MIC of CoQ0 against *C. sakazakii* ATCC 29004 was reported to be 0.1 mg/mL, whereas CoQ0 at 4 mg/mL led to reduction of biofilm formation by the test strain (Guo et al., 2020)

2.5. Inhibition of biofilm formation by C. sakazakii

The process of biofilm formation begins with the preconditioning of the surface by usually electrostatic interactions followed by initial cell attachment, adhesion, retention and proliferation often mediated by Acyl homoserine lactones (AHL) based quorum sensing mechanisms (Whitehead & Verran, 2015). Biofilm formation by pathogens involves

an early reversible attachment stage followed by an irreversible attachment stage. Accordingly, the anti-biofilm agents may act at either stage of biofilm formation or on mature biofilms (Simões, Simões, & Vieira, 2010). Amalaradjou and Venkitanarayanan (2011) reported that anti-biofilm activities of transcinnamaldehyde (TC). The proteomic analysis in the study revealed that the addition of TC disrupted the metabolic pathways of carbohydrate, amino acid and lipids. Additionally, TC interfered with motility, attachment and invasion abilities of *C. sakazakii* (Amalaradjou & Venkitanarayanan, 2011). Biofilm forming *C. sakazakii* strains are reported to release quorum sensing (QS) molecules known as *N*-acyl-*L*-homoserine lactones (AHLs) (Lehner et al., 2005). Different types of AHLs are known to be produced by *C. sakazakii* (da Silva Araújo, Esper, Kuaye, Sircili, & Marsaioli, 2012; Singh, Patil, Prabhune, Raghav, & Goel, 2017). The inhibition of AHL mediated QS has been reported in few studies as potential target to inhibit biofilm formation. Elucidating the anti-quorum-sensing potential of nine plant extracts, Singh, Patil, Prabhune, and Goel (2016) validated the biofilm inhibition of *C. sakazakii*. The methanolic crude extract of *Piper nigrum* and *Cinnamomum verum* showed 75% inhibition of biofilm at a dose concentration of 100 ppm. Eugenol and cinnamaldehyde were suggested as the major bioactive compounds in *Piper nigrum* and *Cinn. verum* respectively for quorum quenching activity. In an another study conducted using thymoquinone (the main active ingredient in the volatile oil of *Nigella sativa* seeds), caused marked inhibition of many virulence related traits such as quorum-sensing (~45% inhibition at a concentration of 150 μmol/L), biofilm formation (inhibited up to 68% after 72 h at a concentration of 600 μmol/L), motility (reduced by ~ 37% at a concentration of 600 μmol/L), endotoxin production (~19% less production at a concentration of 600 μmol/L), cell adhesion and invasion to HT-29 cells and survival in RAW-264.7 macrophages at sub-inhibitory concentration (100 μmol/L) when compared to control (Shi et al., 2017). Thymoquinone was found to suppress transcription of 16 virulence related genes (*fliD, flhD*, *flgJ, ompA, ompX, uvrY, motA, motB*, *sod*, *bcsA*, *bcsG*, *lpx, wzx*, *luxR*, *galE* and *kpsT*). The study also suggested that biofilm inhibition by TQ in *C. sakazakii* was due to the inhibition of genes involved in the production of flagella and cellulose (Shi et al., 2017). TQ has also been reported to inhibit the acid, heat, desiccation, and osmotic stress tolerance of *C. sakazakii* with increased sensitivity to ampicillin and cefoxitin (Chen et al., 2020). Another study reported the anti-biofilm activities of CFS from potential probiotic isolates of goat milk origin against *C. sakazakii* (Singh et al., 2020). A recent review on the strategies to control the biofilm formation by *C. sakazakii* emphasized on the technologies to modify the contact surfaces through surface coatings, use of QS blocking compounds to limit the biofilm formation as future approaches to prevent and control biofilms (Ling et al., 2020).

2.6. Food grade organic acids

Organic acids due to their solubility, taste and low toxicity are among the extensively used food additives and preservatives to prevent food contamination (Table 6). Back, Jin, and Lee (2009) investigated the effect of various organic acids on growth of 51 *C. sakazakii* strains. Among the tested organic acids, propionic acid and acetic acid with a MIC of 16–31 mM and 31–63 mM were the most effective organic acids to control the pathogen in liquid media. Further, the authors reported the inhibitory activities of these two acids in different food matrices such as baby foods and juices. The supplementation of acids in juices enhanced the antimicrobial action of the organic acids (Back et al., 2009). Similarly, Zhu, Schnell, and Fischer (2013) reported the influence of pH of food matrix whereby supplementation of organic acids to slightly acidified PIF indicated a significant reduction of the pathogen population at gastric pH (5.0). The significant observation on the use of acid adapted strains; Zhu et al. (2013) suggested that in determining the antimicrobial actions of organic acids, the adaptation of indicator strains to acidic conditions must be pursued before reaching to any conclusions.

Shi, Song et al. (2016), Shi, Sun, Zhang, Zheng, Yang et al. (2016), and Shi, Sun, Zheng, Zhang, Song et al. (2016) investigated the antibacterial activity of lipoic acid, syringic acid and ferulic acid (FA) against *C. sakazakii* strains. The MIC of all the three acids against *C. sakazakii* strains varied from 2.5 to 5.0 mg/mL. As an antimicrobial activity, reduction of intracellular ATP concentration, pH and cell membrane hyperpolarization were observed. The acids affected the membrane integrity of *C. sakazakii*, as evidenced by a decrease in intracellular ATP concentration. Moreover, the reduction of intracellular pH and cell membrane depolarization was detected in *C. sakazakii* after exposure to these acids. The diminution of ATP concentration, internal pH might be owing to extreme ATP membrane permeability of the tested bacteria caused by these acids through defective membrane leakage of cellular ATP and sustained hydrolysis by the proton pumping ATPase (Gonzalez et al., 1996). The antimicrobial activities of all the three tested acids are reported to be dependent on the functional groups present in these and their lipophilic characteristics (Shi, Sun, Zheng, Zhang, Song et al., 2016). Since these organic acids are food grade acids, therefore, further studies investigating their inhibitory activities in different food matrices and their effects on the organoleptic properties of foods are required prior to their commercial applications.

2.7. Synergistic activities

Considering the strain specific resistance abilities of *C. sakazakii* to environmental stresses (Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015; Dancer, Mah, Rhee, Hwang, & Kang, 2009; Healy et al., 2010), synergistic approach using natural antimicrobials with other biological, chemical and physical agents at mild level are suggested as a better alternative to eliminate the *C. sakazakii* (Martín-Belloso & Sobrino-López, 2011; Kent et al., 2015) (Table 7). Most of the antimicrobial organic acids had issues with their solubility; therefore, a few studies employed temperature treatment in conjunction with the organic acid to enhance their solubility and associated antimicrobial activities. To check enhancement if any of mild heat treatment on anti-*Cronobacter* activity of caprylic acid (an eight carbon fatty acid; found in mammalian milk and act via membrane disruption), Jang and Rhee

Table 6
Antimicrobial activities of food grade organic acids against *C. sakazakii*.

Organic acid	Protection mechanism	Targeted strain	Reference
Malic acid, lactic acid, formic acid, phosphoric acid, propionic acid, citric acid, tartaric acid, acetic acid	Not specified	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC 12868 and 71 other strains of *C. sakazakii*	Back et al., 2009
Malic acid, lactic acid, butyric acid, propionic acid, citric acid, tartaric acid, acetic acid	Synergistic effect of organic acid and physiological infant gastric pH	30 Food and clinical *C. sakazakii* strains	Zhu et al., 2013
Ferulic acid, Syringic acid, Lipoic acid, citral	Cell membrane disintegration	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC BAA-894, *C. sakazakii* ATCC12868 and five other *C. sakazakii strains*	Shi, Song et al. (2016), Shi, Sun, Zhang, Zheng, Yang et al. (2016), Shi, Sun, Zheng, Zhang, Song et al. (2016)

Table 7
Biological methods augmented with physical and chemical approaches against *C. sakazakii*.

Synergistic method	Protection mechanism	Targeted strain	Reference
Caprylic acid (30mMol) and mild heat (45–55 °C)	Cell membrane disruption	*C. sakazakii* strains	Jang & Rhee, 2009
Lactic acid (0.2%) and copper- II (50 μg/ml)	Not specified	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC 12,868 (10^6 CFU/ml)	Al-Holy et al., 2010
Propionic (0.1%) and monocaprylin (0.2%) Lactoperoxidase system (×2) and lacticin 3147		*C. sakazakii* ATCC 29544 and nine clinical *C. sakazakii* isolates (10^2 CFU/ml)	Oshima et al., 2012
Monocaprylin (0.2 mg/ml) and high temperature (50 °C)	Cytoplasmic leakage	*C. sakazakii*DBM 3157	Marounek et al., 2012
Cocoa powder (5%) in combination with pulse electric field (15 kV/cm)	Not specified	*C. sakazakii* strain	Pina-Pérez, Martínez-López, & Rodrigo, 2013
Combination of Trans-cinnamaldehyde (0.05%), Chitosan (1%) and high pressure processing (600 MPa for 5 min.)	Not specified	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC BAA-894, *C. sakazakii* ATCC 12868	Cetin-Karaca, 2015
Nisaplin (food grade formulation containing Nisin A) in combination with citric acid	Not specified	*C. sakazakii* NCTC 8155::p16*Slux*-P_{help}	Campion et al., 2017
Peroxyacetic acid (200 ppm) and ultrasound (37 kHz, 380 W for 60 min)	Not specified	*C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC 12868	Bang et al., 2017

(2009) added caprylic acid to PIF at different doses followed by mild heat treatments (45–55 °C for different time intervals). A rapid reduction in log CFU (~7 log reduction at a concentration of 30 mM at 55 °C within 10 min) was reported due to synergistic activity. The enhanced antimicrobial effect was suggested due to increased solubility of caprylic acid with increasing temperature and disruption of the integrity of a cell membrane. Similar results were also obtained by Marounek, Putthana, Benada, and Lukešová (2012) for monocaprylin (0.5 mg/mL) at 50 °C, as the treatment resulted in two fold decrease in *C. sakazakii* count and was suggested as a method of choice for *C. sakazakii* inactivation. Choi, Kim, Lee, and Rhee (2013) have reported the synergistic activity of caprylic acid, citric acid and vanillin in spiked reconstituted PIF. The combination of caprylic acid (20 mM) and citric acid (30 mM) reduced the log CFU count of *C. sakazakii* by more than 7.3 log CFU/mL within a contact time of 5 min at 40 °C. A dose dependent effect of triglycerol monolaurate (TGML) has also been reported recently by Zhang et al. (2020) whereby the enhanced action of TGML was reported at lower pH. The enhanced activity of these selected acids was suggested due to enhanced solubility at the mild temperatures. The combination resulted in plasmolysis and membrane disintegration due to synergistic action.

To investigate the combined effect of polyphenol-rich cocoa powder (CocoanOX 12%, CCX) and Pulsed Electric Field (PEF) on the susceptibility of *C. sakazakii*, Pina-Perez et al. (2013) added different concentration of cocoa powder to *C. sakazakii* spiked PIF. The optimization of cocoa powder dose was done at a different time interval before and after PEF treatment at 8 °C. The study concluded that maximum inhibition (by ~4.4 $\log_{10}$) was achieved when CCX (5%) was added after 4 h of PEF treatment (15 kV/cm for 3000 ms) and stored under the refrigerated condition for 12 h. The observed synergy may be attributed to sensitization of *C. sakazakii* due to sublethal injury induced by PEF which further enhanced the injury by CCX.

Using lactic acid (LA) in combination with copper (II) has been studied against the *C. sakazakii* by Al-Holy, Castro, and Al-Qadiri (2010). Study concluded that both the agents at their sublethal concentration i.e., 0.2% and 50 μg/mL respectively eliminated *C. sakazakii* completely from reconstituted infant formula (RIF) and PIF with enhanced antibacterial effect as compared to effect exerted by each agent, individually. The observed synergy was speculated due to the permeabilization effect exerted by LA that facilitated copper ion penetration through the cell membrane. After entering into the cell, copper ion may either inactivate enzyme or obstruct functional groups of protein, generating free radicals that ultimately affects membrane integrity or may interfere with nucleic acid and protein conformation, interfering with oxidative phosphorylation and osmotic balance.

In order to search for anti-*Cronobacter* agents which would work efficiently even at mild reconstitution temperatures and longer storage period in reconstituted PIF, Oshima et al. (2012) screened thirty-three antimicrobial agents, including organic acids, bacteriocins and lactoperoxidase system for their efficacy against *Cronobacter* spp. Among tested antimicrobials, a combination of propionic acid and monocaprylin (at a dose of 0.1% each) reduced the microbial population by 50%. The addition of bacteriocins (nisin, lacticin) to this combination did not cause any further reduction in bacterial viable counts, however, the supplementation of bacterioicins with LPOS resulted in 50% reduction in the population of *C. sakazakii*. The authors also suggested the use of a combination of LPOS and bacteriocins as these are generally regarded as safe by WHO/FAO (2006).

Cetin-Karaca (2015) reported the enhanced antimicrobial efficacy of TC (0.05%), chitosan (1%) in combination with high pressure processing (HPP) against *C. sakazakii* in PIF. The combination reduced the *C. sakazakii* count by 7.3 log CFU/mL after 4 h of storage. The transmission electron microscopy (TEM) analysis indicated the deformations in the cell wall of organism and leakage of cellular contents as bactericidal mechanisms without any changes in organoleptic characteristics of PIF. Campion et al. (2017) established the enhanced combinatorial effect of nisin derivatives with EO's (thymol, trans-cinnamaldehyde and carvacrol) and citric acid. Engineered nisin (S29A) exhibit almost two-fold specific activity against *C. sakazakii* as compared to wild type nisin A; however, another nisin derivative nisin V possessed similar activity as that of nisin A. An enhanced activity of nisin peptides were observed when combined with plant essential oils (thymol, carvacrol, trancinnamaldehyde at therate of ~4 fold, 2 fold and 1.5 fold, respectively). Despite that, nisin and nisin derivatives in combination with essential oil failed to inhibit *C. sakazakii* in PIF; however, food grade Nisaplin in combination with citric acid successively reduced *C. sakazakii* by ~3 fold in reconstituted PIF. Altogether these findings suggested combined usage of nisin and essential oils to reduce the antimicrobial dosage and undesirable nutritional and organoleptic properties. Recently, the transcriptomic studies revealed that a dose dependent combination of citral and carvacrol caused disruption of inner membrane and dissipation of proton motive force of *C. sakazakii* CICC 21544 (Cao et al., 2020).

An exposure of ultrasound (cavitation agent) for 60 min in combination with 150–200 ppm peroxyacetic acid (antibacterial agent) was suggested for the biofilm inhibition on fresh produce, as it does not affect the colour, texture, chewiness and moisture content while effectively removing biofilm from the surface (Bang, Park, Kim, Rahaman, & Ha, 2017). The combination was reported to reduce the bacterial load by 3.51 log CFU/mL as compared to individual reduction levels of 0.04–0.60 and 0.89–1.88.

Using this synergistic approach, Turňa (2015) evaluated the anti-*Cronobacter* activities of cathelicidin LL-37 (a human origin antimicrobial peptide) alone, and in combination with bacteriophage Dev-

CS-701. The study indicated that a higher concentration of LL-37 (50–100 μg/mL) was required to reduce *C. sakazakii* population substantially (70–96%) in PIF as compared to liquid growth medium thereby suggesting significant effect of food matrix upon observed activity as LL-37 being positively charged may exert a reduced efficacy due to its interaction with negatively charged compounds. Therefore, the authors further checked the efficacy of a combination of LL-37 with bacteriophage in reconstituted PIF. A reduction of 1300 fold in *C. sakazakii* population was observed with this combination, showing a significant level of synergy. The combined application of bacteriophage with LL-37 used as a lower dose of bacteriophage and reduced the chances of phage-resistant cell generation.

3. Future perspectives and suggestions

In this review, we have discussed several biological methods which are the most diverse, emerging and interesting area of research towards the control of foodborne pathogens such as *C. sakazakii*. Most of the studies reviewed, focused on the application of biocontrol agents individually or in synergy with other physico-chemical methods were performed in reconstituted PIF. There is a long list of the biological molecules and methods that showed the potential antimicrobial activity against *C. sakazakii* in the laboratory conditions. There is limited information about the application of these methods in food processes depicting that product formulations using these agents are still a challenge to the infant food manufacturers. An improvement in the processing or composition of infant food products as per standard guidelines could be a great promising area of further research for the effective control of *C. sakazakii*.

Bacteriocins have been proved to have promising activity against *C. sakazakii*, however, it is a challenging task to maintain the stability of bacteriocins during the processing of the dehydrated food products. Therefore, application of encapsulation methods to control the release of bacteriocins during the processing of food products and thereafter could be an area of further research. In addition to bacteriocins as secretory components of probiotics, the cell surface components or their combinations referred to as postbiotics need to be investigated for their effect against foodborne pathogens. Whereas the use of combination of different natural substances such as polyphenols and other plant products might help in reducing the dosages for effective bactericidal components and the effect of these substances on sensory qualities of foods needs to be explored. A combination of the novel food decontamination processing methods such as high-hydrostatic pressure, sonication, microwave, irradiation (UV-C treatments), ohmic heating, ozonation, pulsed electric fields, and cold plasma along with the combination of biological methods might be a promising approach for controlling the foodborne pathogens including *C. sakazakii* in food commodities. The biological methods are considered as one of the effective and best method for the control of bacterial pathogens in several food products due to the emergence of antimicrobial resistance in the pathogens as well as these are considered safe for human consumption.

From the industrial point of view, the cost for the utilization of above discussed biological methods is an additional critical aspect for their adoption and implementation in any food industries. Most of the research carried out towards the control of *C. sakazakii* has not explored the production cost as compared to the conventional approaches. The cost of method and its efficiency in food processing are important considerations in the acceptance of products manufactured with this technology. It is obvious that research and development in the area of food safety can overcome the technical difficulties and production cost of these methods as well as penetration of the products in market. However, it is not explored yet in the present case. The maintenance of organoleptic properties such as flavor, colour, taste and texture in addition to compositional changes in manufacture of infant formulae is another critical aspect in controlling the contamination of *C. sakazakii*.

Most of the potential risks that can be associated with the use of these biological control approaches need to be explored in different food matrices such as their toxicity, stability at food processing conditions, interaction with other food components. Further, the dose of the specified biological control agent needs to be optimized as per the requirement and standards of the infant food product formulations.

4. Conclusions

Despite of various advances in food processing technologies, complete elimination of foodborne pathogens from food commodities is one of the most challenging tasks. Co-currently, increased consumer demand for safe and high quality food products with minimal physicochemical processing has driven the food scientists and technologists to search for alternative biological methods for the control of pathogens. As per the literature presented in the reivew, no single risk mitigation measure can achieve the complete inhibition of *C. sakazakii* in terms of dose and mode of action which emphasizes the use of a combination of mitigation measures, or hurdles, to control and/or eliminate the pathogen or its biofilms. The synergistic action of these agents indicate higher efficacy which needs to be investigated further for their suitability in different food matrices other than infant formula. The novel approaches such as nanotechnology based delivery mechanisms with active functional molecules should also be investigated in overcoming the biofilm formation by the pathogen in the food processing equipments. Further, an authorization from the health authorities is required for the implementation of such technologies at the industrial level.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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