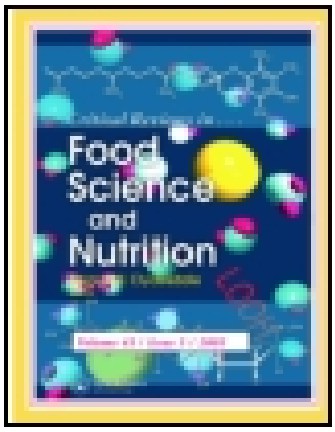

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Non-Thermal Inactivation of Cronobacter Sakazakii in Infant Formula Milk: A Review

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Non-thermal inactivation of *Cronobacter sakazakii* in Infant Formula Milk: a review

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Abstract

Up-to-date, non-thermal technologies and combinations of them, in accordance with the "hurdle technology" concept, are being applied by different research groups in response to calls by the International Food and Human Health Organizations (ESPGHAN, 2004; FAO/WHO 2006, 2008) for alternatives to thermal control of *Cronobacter sakazakii* in reconstituted powdered infant formula milk (RPIFM). This review highlights (i) current knowledge on the application of non-thermal technologies to control *C. sakazakii* in infant formula milk; and (ii) the importance of the application of non-thermal technologies for the control of *C. sakazakii* as part of the development of strategies in the context of improving food safety and quality of this product.

Keywords: powder infant formula milk (PIFM), *Cronobacter sakazakii*, non-thermal technologies, infant feeding.

Introduction

Powdered infant formula milk (PIFM) has been the most common vehicle implicated in *Cronobacter sakazakii* infections: life-threatening forms of neonatal meningitis, sepsis and necrotizing enterocolitis (NEC) in newborn and premature infants (Friedemann, 2007). To avoid the public health risk of possible contamination of PIFM, FAO/WHO (2006) recommends reconstitution of PIFM at temperatures ≥ 70 °C. In these conditions, Osaili et al. (2009) achieved a 5.3 $\log_{10}$ cycle *C. sakazakii* inactivation. However, undesirable effects on the organoleptic, nutritional and functional properties of thermally treated products have been extensively described (Barbosa-Canovas & Aguirre, 2010). To improve RPIFM nutritional quality to meet the specific health requirements of the neonatal and premature population and guarantee food safety, non-thermal technologies for *Cronobacter spp.* inactivation have been proposed. Since 2006, various research groups have been actively working on a number of non-thermal technologies applied to *C. sakazakii* control specifically in reconstituted PIFM (RPIFM)

High Hydrostatic Pressure Processing (HPP)

The potential application of this technology to *C. sakazakii* inactivation is focused on the process of wet mixture treatment before spray drying (Koseki, Matsubara, & Yamamoto, 2009), and the production of a novel non-thermally treated pasteurized liquid infant milk formula (Pina-Pérez et al., 2007a).

The first study on *C. sakazakii* inactivation by HPP was conducted by González et al. (2006) in reference medium and RPIFM. Inactivation levels between 2 and 6 $\log_{10}$ cycles were achieved. Novel HPP studies of *C. sakazakii* inactivation have been conducted by Pina-Pérez et al. (2007a) and Arroyo et al. (2011a). Maximum inactivation levels ranged between 5 to 7 $\log_{10}$ cycles under

different treatment conditions. Also, in both works a clear substrate effect on HPP inactivation levels achieved was observed, the cells being more sensitive to pressure in reference medium, possibly owing to the baroprotective intervention of real matrix constituents (e.g. fats and proteins in RPIFM) (Pina-Pérez et al., 2007a; Arroyo et al., 2011a).

Pina-Pérez et al. (2007a) pointed out that at relatively low pressure levels (100–200 MPa), increases in holding time of 10–20 min were not translated into enhancements of inactivation levels, with tails appearing in survival curves. On the basis of inactivation kinetics obtained by Koseki and Yamamoto (2007), Koseki, Matsubara, & Yamamoto (2009) developed a probabilistic model for predicting *Cronobacter spp*. inactivation by HPP (P = 400–600 MPa; t = 1–20 min; temperature 25–40 °C; inoculum levels 3, 5, 7 $\log_{10}$) in reference medium and RPIFM. This model is the first stochastic model to predict microbial load levels and reductions and their associated probability of occurrence. Description of non-thermal process effectiveness in stochastic terms is of great importance, contributing to exposure assessment and risk management processes (Pina-Pérez et al., 2012; Poschet et al., 2005).

Pulsed Electric Fields (PEF)

FAO/WHO (2008) have recognized the need to develop novel alternative technologies to control and inactivate this pathogenic microorganism, specifically in hospital settings, because of the fatal consequences associated with it for the neonatal population (Van Acker et al., 2001). To meet international safety requirements, it has been proposed that PEF technology should be used in hospital settings just after PIFM reconstitution as an alternative for *Cronobacter spp*. control,

and that PEF-treated RPIFM should be handled according to Codex Alimentarius Commission guidelines (CAC, 2008).

The first study on PEF control of *Cronobacter spp.* was carried out by Pina-Pérez et al. (2007b) using 0.3 % buffered peptone water (w/v) and RPIFM substrates. According to Pina-Pérez et al. (2007b), a maximum inactivation level of 1.20 $\log_{10}$ cycles was achieved in RPIFM at 40 kV/cm-360 μs, 2.70 $\log_{10}$ cycles being the maximum level achieved in reference medium at the same conditions. The influence of the substrate is consistent with the substrate influence results of other authors working with other microorganisms (Knorr, 2001; Evrendilek, Zhang, & Richter, 2004). Recently, novel studies on PEF inactivation of *Cronobacter spp*. have been performed by Arroyo et al. (2010a) in citrate-phosphate buffer, achieving a maximum inactivation level of 5.1 $\log_{10}$ cycles ((31 kV/cm; 50 pps) (pH = 7)). The study conducted by Arroyo et al. (2010a) reveals the influence of environmental factors (pH, a_w, temperature and incubation time growth conditions [T, t]) on inactivation, indicating increased *C. sakazakii* PEF tolerance under acidic conditions (pH = 4) and in low water activity media (a_w = 0.97).

Arroyo et al. (2010a) and Pina-Pérez et al. (2007b) agree that the PEF process parameters, electric field strength (E, kV/cm) and treatment time (t, μs), have a significant influence on microbial inactivation, the survival curves being close to linearity or deviating slightly, depending on the E applied.

Taking into account the widely recognized mechanism of inactivation by PEF (electroporation), special attention is being paid to the possible generation of sublethally damaged cells which could represent a risk during refrigerated storage of treated RPIFM. Arroyo et al. (2010b)

measured membrane integrity loss by increased uptake of the fluorescent dye propidium iodide (PI). They found a pH effect on sublethal injury generation by PEF (25 kV/cm-100 pulses), the percentage of injured population (99.5 %) being higher, at the cytoplasmic and outer membrane level, under acid treatment conditions (pH = 3.5–4) than under neutral ones (pH = 5–7). However, 43 % of this injured population (3 $\log_{10}$ cycles) had largely recovered after 4 h at room temperature. In the sublethal damage study by Pina-Pérez et al. (2009), the percentage of injured cells achieved in RPIFM (pH = 6.4) was up to 90 % at the lowest electric field strength, 15 kV/cm-30000 μs, doubling the inactivation levels after 24 h of storage (8 °C).

This technology could provide an effective additional control measure to be applied in hospital settings using compact equipment under optimized treatment conditions, contributing to reduce contamination levels at the time of consumption below infectious dose 10^3 CFU (fao/who, 2004).

Ultrasound

Some authors have proposed the use of ultrasound waves (UW) as a food preservation method for *Enterobacteriaceae* inactivation (Le, Kermasha, & Baker, 1989; Wrigley & Llorca, 1992). Cavitation has been recognized as being responsible for the bactericidal effect of UW (Raso et al., 1998).

Adekunte et al. (2010) and Arroyo et al. (2011b, c) have carried out the research studies on this subject that have been published to date. Owing to the limited lethal effect of ultrasound technology on its own, according to several authors (Guerrer et al., 2001; Zenker, Heinz & Knorr., 2003), UW has been used in combination with temperature (thermosonication)

(Adekunte et al., 2010) or pressure (manosonication) (Arroyo et al., 2011b), or pressure and heat (manothermosonication) (Arroyo et al., 2011c).

With respect to heat-UW effectiveness against *C. sakazakii*, maximum inactivation levels in the range [6.86–7.05] $\log_{10}$ cycles at 50 °C-61 µm (2.5 min) have been achieved by Adekunte et al. (2010) in RPIFM. With regard to process conditions, the higher the wave amplitude (24.4 to 61 µm), the higher the inactivation of *C. sakazakii* achieved at each temperature (Adekunte et al., 2010). Survival curves obtained for *C. sakazakii* inactivation by ultrasound proved to be linear and biphasic in RPIFM, depending on temperature and wave amplitude (25–50 °C, 24.4 to 61 µm) (Adekunte et al., 2010).

Arroyo et al. (2011b) studied the effect of UW (34, 62, 90, 117 and 145 µm) under pressure (manosonication, MS) (0, 50, 100, 200 and 300 kPa). The D_{MS} value (decimal reduction time value) of *C. sakazakii* in standard conditions (35 °C, 117 µm, 200 kPa, citrate-phosphate buffer pH 7.0) was 0.41 min. An exponential relationship was found between ultrasonic wave amplitude and D_{MS} values, but the relationship between static pressure and D_{MS} values was better described by a quadratic equation. Arroyo et al. (2011c) and Adekunte et al. (2010) reported the dependence of UW inactivation on temperature increase: the higher the temperature, the higher the inactivation achieved by UW. Arroyo et al. (2011c) emphasized the increase in levels of inactivation by UW at 45–64 °C, indicating a synergistic effect of this hurdle combination.

Advantages of ultrasound include reduced processing time, higher throughput and lower energy consumption, and therefore specific UW equipment could be developed for hospital applications, to process RPIFM before feeding infants. On the basis of the results obtained to date concerning the resistance of *C. sakazakii* to UW, treatments should be validated previous to its scale-up at

industrial level, in order to develop effective pasteurization and sterilization processes using this technology, taking into account that there are not equipments available for commercial use.

Gamma and electron-beam radiation

Irradiation using an electron beam without the intervention of any radioactive source is widely recognized as a non-health-compromising technology which has recently been applied to preserve various food products (Chung, Ko, & Kin, 2000; Tarte, Murano & Olson, 1996; Van Calemberg et al., 1999; Nieto-Sandoval et al., 2000; Kwon, Lee, & Kwon 2001). According to the FAO/IAEA/WHO Expert Committee on Food Irradiation report, "the irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard" (WHO 1981).

With regard to *C. sakazakii* control in powdered infant formula milk, a great advantage of this ionizing irradiation technology is its possible application directly to the dehydrated product. Hong et al. (2008), Lee, Kermasha, & Baker (2007) and Osaili et al. (2007) reported the effectiveness of both electron-beam and gamma irradiation, respectively, to control *C. sakazakii* in PIFM. *C. sakazakii* showed higher resistance (than *Bacillus cereus* and *Salmonella Typhimurium*) to electron-beam irradiation, with decimal reduction dose (D_{10}) value = 4.83 (Hong et al., 2008) in a powdered weaning food formulated from infant formula containing rice and rice flour. According to studies of regrowth of treated *C. sakazakii* cells in RPIFM, it was observed that samples irradiated at 2 and 8 kGy grew by up to 5.30 and 3.50 $\log_{10}$ cycles, respectively, while non-irradiated samples increased by 6.20 $\log_{10}$ cycles after 12 days of storage.

Lee, Kermasha, & Baker (2007) and Osaili et al. (2007) also reported the effectiveness of gamma radiation to control *C. sakazakii*. Gamma irradiation processes were carried out at 10 ± 0.5 kGy at room temperature (20 ± 0.8 °C). Lee, Kermasha, & Baker (2007) revealed the high sensitivity of *C. sakazakii* to gamma radiation, 5 kGy being necessary to completely eliminate the pathogen inoculated at 10^8–10^9 cfu/g in PIFM. In view of the concern about possible regrowth of *C. sakazakii* in reconstituted product, Lee, Kermasha, & Baker (2007) carried out a post-treatment storage study (10 °C), finding that, irrespective of treatment dose (1, 3, 5 kGy), no growth was observed until 6 h.

Osaili et al. (2007) reported *C. sakazakii* sensitivity to gamma irradiation, achieving up to 7 $\log_{10}$ cycles reduction at a dose of 8 kGy, with a D_{10} value of 0.29 ± 0.03. The sensitivity of this microorganism in PIFM was found to be similar to that of *E. coli* O157:H7 (Wang Reitmeier & Glatz, 2004).

In agreement with the response of *C. sakazakii* to other non-thermal technologies, the microbe's sensitivity to irradiation is conditioned by substrate composition, inactivation levels achieved in reference medium being considerably higher than those achieved in RPIFM (Osaili et al., 2007; Lee, Kermasha, & Baker, 2007). According to the irradiated RPIFM reported results, and the EFSA opinion (EFSA, 2011) regarding irradiated foods, 6 kGy would be enough to reduce several $\log_{10}$ units of foodborne pathogens in dried products, and consequently to control *C. sakazakii* in powdered infant formula milk.

Antimicrobials

Various natural substances have been proved to be bacteriostatic or bactericidal against *C. sakazakii* in reconstituted powdered infant formula milk: (I) Caprylic acid (Nair et al., 2004; Jang and Rhee, 2008); (II) *trans*-cinnamaldehyde) (Amalaradjou, Hoagland, & Venkitanarayanan, 2009); (III) polyphenol-rich cocoa powder (Pina-Pérez et al., 2011), and (IV) bovine lactoferrin (LF) and nisin (Al-Nabulsi & Holley, 2007). Moreover, carvacrol, thymol, eugenol, diacetyl, cinnamic acid (Lee and Jin, 2008) and water soluble muscadine seed extracts (Kim et al., 2009) have been tested against *C. sakazakii* in reference medium (tryptic soy broth, TSB).

Nair et al. (2004) and Jang and Rhee (2008, 2009) studied the effect of monocaprylin and caprylic acid, respectively, against *C. sakazakii*. Caprylic acid (octanoic acid) (GRAS 184.1025) has been associated with antimicrobial and immunomodulatory activities (Baranyi, Thomas, & Pellegrini, 2003; Sprong et al., 2001). Initial *C. sakazakii*-contaminated RPIFM (7.7 $\log_{10}$ cycles) was treated at room temperature at different caprylic acid concentrations (5, 10, 20 and 30 mM) (Jang and Rhee, 2009). A reduction of around 7.8 $\log_{10}$ cycles was observed after 1 h (45 ºC) as a result of addition of 30 mM caprylic acid to RPIFM. Moreover, antivirus properties have been described for free fatty acids and monoglycerides, with a corresponding beneficial effect resulting from their addition to RPIFM because of their possible protective effect for premature infants against syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), *Haemophilus influenzae* and Group B *Streptococcus*.

Treatments with monocaprylin at 0.25 and 50 mM were carried out at 4, 8, 23 and 37 °C. Inactivation levels were dependent on both monoglyceride concentration and incubation temperature. The microbial population was reduced by 2 and 6 $\log_{10}$ cycles after 1 h with 25 and

50 mM monocaprylin addition, respectively, to RPIFM at 37ºC; and up to 4 and 5 $\log_{10}$ cycles at 23 ºC. *C. sakazakii* was reduced below detection limits (> 5 $\log_{10}$ cycles) after 24 h of incubation at 8–4 °C as a result of monoglyceride addition (25 and 50 mM).

Trans-cinnamaldehyde is a major component of cinnamon bark extract. It is classified as generally recognized as safe (GRAS) by the FDA, and is approved for use in foods (21 CFR 182.60). To consider the possible effectiveness of *trans*-cinnamaldehyde against *C. sakazakii* (10^7 cfu/mL), 0 % (control), 0.15 %, 0.3 % and 0.5 % (w/v) concentrations were tested. After 24 h of incubation of inoculated RPIFM, no detectable levels were found in samples supplemented with 0.3 % of *trans*-cinnamaldehyde, with 3.5 $\log_{10}$ cycles remaining in samples treated with 0.15 % of *trans*-cinnamaldehyde. Similar reduction levels were observed at 23 °C (24 h) in the presence of *trans*-cinnamaldehyde (0.15, 0.3 and 0.5 %). Control samples at these temperatures increased the *C. sakazakii* load by up to 9.5 $\log_{10}$ cycles after 24 h, however, at 8-4 ºC, the population of *C. sakazakii* after 24 h of storage did not vary significantly. An enhancing inactivation effect was observed at these temperatures, the pathogen being completely inactivated by 0.5 % *trans*-cinnamaldehyde after 10 h (8–4 °C). The marked increase in inactivation levels attributed to the contribution of low temperature (8–4 °C) was possibly due to changes in the fatty acid profile and fluidity of the cell bacterial membrane at cold temperatures (Amalaradjou, Hoagland, & Venkitanarayanan, 2009).

Lactoferrin (LF) and nisin are natural antimicrobial substances extensively reported to control food pathogens (Farnaud & Evans, 2003; Barbiroli et al., 2012). According to Al-Nabulsi et al. (2009), the study of LF and nisin effect to control *C. sakazakii* was performed in 0.2 % peptone water and RPIFM. Samples in the presence of LF were incubated at 37, 21 or 10 °C, while those

containing nisin were incubated at 37 or 21 °C. According to Al-Nabulsi et al. (2009), the effectiveness of LF, in 0.2 % peptone water, against *C. sakazakii* was temperature-dependent, being reduced at lower temperatures. Reduction levels of at least 5 $\log_{10}$ cycles were achieved in the presence of LF at ≥ 2.5 mg/mL (37 °C, ≥ 4 h); at ≥ 2.5 mg/mL (21 °C, ≥ 8 h); at ≥ 10 mg/mL (10 °C, ≥ 48 h). With regard to the effect of nisin in 0.2 % peptone water, reduction levels ≥ 5 $\log_{10}$ cycles were achieved at the following conditions: nisin addition ≥ 1500 IU/mL (37 °C, ≥ 4 h); nisin addition ≥ 1500 IU/mL (21 °C, ≥ 8 h). However, the effects of both LF and nisin proved to be substrate-dependent, inactivation levels in RPIFM being undetectable at the conditions studied. Moreover, the combination of nisin (0, 500, 1000, 1500, 2000 IU/mL) and mild heat did not produce an interaction to reduce *C. sakazakii* in RPIFM.

Polyphenol-rich cocoa powder has been shown by Pina-Pérez et al. (2011) to be bacteriostatic or bactericidal against *C. sakazakii* at 25 °C. Growth curves were fitted to the Gompertz equation and specific growth rate (μ_{max}) and lag time (λ) values were obtained for different concentrations of polyphenol rich cocoa powder (1, 2.5, and 5 % (w/v)) added to infant formula milk. Lag time duration increased significantly ($p \leq 0.05$) as a result of 5 % cocoa addition while μ_{max} values were around 0.350 ± 0.025 ((cfu/mL)/h) in the various beverages. From the results, the authors concluded that polyphenol-rich cocoa powder at the concentration studied has a bacteriostatic effect against *C. sakazakii* when it is added to RPIFM. Other products based on polyphenol antimicrobial capability, soluble muscadine seed extracts, have been studied against *C. sakazakii* in reference medium (Kim et al., 2009). Hot "Carlos" (bronze) and "Ison" (purple) muscadine seed powders reduced *C. sakazakii* to undetectable levels after 30 min and completely after 60 min, mainly owing to polar substances in the extracts, such as tartaric, malic and tannic acids.

Lee and Jin (2008) reported that the classification of natural antimicrobial compounds in accordance with their effect against *C. sakazakii* suspended in reference medium was as follows: carvacrol = thymol > eugenol > diacetyl > cinnamic acid, with a minimal inhibitory concentration (MIC) of 1.25 mmol/L for the strongest inhibitory substances, carvacrol and thymol, and a MIC of 5 mmol/L for the weakest inhibitory substance, cinnamic acid.

Al-Holy, Castro, & Al-Qadiri (2010) treated RPIFM with copper sulphate and lactic acid, alone and in combination. The use of copper sulphate at a concentration of 50 ppm and lactic acid at 0.2 % v/v had a slight but noticeable effect on growth of *C. sakazakii*, with a reduction of 3 $\log_{10}$ after 2 hours and complete inhibition of growth after 6 hours. The results indicate that the combination of lactic acid and copper sulphate could be used for the control of *C. sakazakii* contamination in RPIFM.

Finally, to complete the summary of natural substances with an antimicrobial effect on *C. sakazakii*, mention must be made of the great number of bioactive peptides which have been identified in milk proteins and to which antimicrobial properties have been attributed, such as casein and whey proteins. According to Lahov and Regelson (1996), the antimicrobial peptide isracidin has a broad spectrum of activity (*Staphylococci, Sarcina, Bacillus subtilis, Diplococcus pneumoniae* and *Streptococcus pyogenes*). Hayes et al. (2006) assessed the activities of antimicrobial peptides produced by *Lactobacillus acidophilus* against *Cronobacter spp*. More recently, the activity of a sodium caseinate fermentate for reducing the numbers of *Cronobacter spp.* in reconstituted PIFM was tested (Hayes et al., 2009). At higher final concentrations of 3.33 % (w/v), the initial *C. sakazakii* count was reduced by approximately 6 $\log_{10}$ cycles to undetectable levels over a period of 60 min.

Some natural ingredients with antimicrobial capability against *C. sakazakii* are commonly included in beverage formulations for flavouring purposes. On the basis of our current knowledge, the promising *C. sakazakii* inactivation results obtained by using natural antimicrobials such as cocoa and cinnamon could be focused on the development of new pasteurized beverages for toddlers and children (1–3 years).

Non-Thermal Plasma (NTP)

Joshi et al. (2010) studied the effectiveness of a fluidized bed, non-thermal plasma on two different *Enterobacteriaceae*, *E. coli* K-12 and *C. sakazakii*, in PIFM. Polytetrafluoroethylene (PTFE) beds were selected as a model dry particle, 100 dry inoculated ($9.58 \pm 1.95 \times 10^5$ cfu/cm^2) PTFE beds being added to 10 g of infant formula milk, successfully transferring $2.79 \pm 2.2 \times 10^6$ cfu/g organisms into PIFM. The fluidized bed plasma chamber was fitted over the plasma discharge emitter head of a Dyne-A-Mite variable chemistry plasma unit using compressed air as a primary gas and helium gas as a secondary at a flow rate of 4, 6 and 8 Lpm. Treatment time was 0 (control), 5, 10, 15, 20, 25, 30, 40, 50 and 60 s.

A reduction of 0.12 to 6.94 $\log_{10}$ in the *C. sakazakii* load was achieved at 5 and 30 s respectively, following a linear trend. Treatment at 4 cm from the plasma discharge resulted in a greater reduction and less variation was observed than at a distance of 6 cm. Despite treatment variations, longer duration of treatment almost always resulted in enhanced reductions of *C. sakazakii* and *E. coli* K-12 counts, *C. sakazakii* being more resistant to plasma treatment than *E. coli* K-12. Future research prospects include argon secondary gas use and establishment of D-

values to determine the efficacy of NTP treatment of *C. sakazakii* in powdered infant formula at different frequencies of the power supply.

Bacteriophages

The use of bacteriophages to prevent *C. sakazakii* growth has been investigated by Kim, Klumpp & Loessner, (2007a) and Zuber et al. (2008). Kim, Klumpp & Loessner (2007a) reported the isolation and application of two novel *C. sakazakii* bacteriophages and 5 food isolates (236/04, 732/03, 966/04, 1154/04 and 1156/04) in reference media and RPIFM at 12, 24 or 37 °C.

According to the results of Kim, Klumpp & Loessner (2007), highly purified phage stocks (2 x 10^{11} cfu/mL) were obtained for *C. sakazakii* ATCC 29544 (ESP 1-3) and the food isolate *C. sakazakii* 732/03 (ESP 732-1). The bacteriophages obtained exhibited a high host specificity, ESP 1-3 being able to lyse both *C. sakazakii* ATCC 29544 and *C. sakazakii* 732/03. ESP 732-1 proved more effective against *C. sakazakii* growth at different temperatures, with bacteriophage effectiveness proving to be concentration-dependent. The higher the bacteriophage concentration (10^7–10^9 PFU/mL), the higher the inhibition effect observed in reference medium and RPIFM. However, with regard to temperature influence, 24 °C was the most effective temperature to inhibit *C. sakazakii* after 16 h. In RPIFM, the *C. sakazakii* population was reduced by 8 $\log_{10}$ cycles to undetectable limits after 2 h and no growth was observed during a period of 14 h for both ESP 1-3 and ESP 732-1 bacteriophages.

Zuber et al. (2008) isolated 67 phages from environmental samples and tested their lytic host range on a representative collection of 40 *C. sakazakii* strains. A high dose of 10^8 pfu/mL of phage could effectively sterilize a broth contaminated with both high and low pathogen counts

(10^6 and 10^2 cfu/mL). After inoculating 10^4 pfu/mL and 10^2 cfu/mL of bacteria in broth, it was observed that it was only when the concentration of bacteria crossed the 10^5 cfu/mL threshold that the phages started to intervene with microbial reduction, but they did not reduce the count below 100 cfu/mL.

According to the EFSA report (2009) concerning bacteriophages in food production, specific bacteriophage-pathogen-food combinations should be developed, maximizing bacteriophage effectiveness as a decontaminating method. There is a remarkable potential for applying bacteriophages in the disinfection of the PIFM manufacturing environment

Supercritical carbon dioxide

Supercritical carbon dioxide (SC-CO_2) exists beyond a critical point (7.38 MPa and 31.1 °C), and has been used extensively for extraction purposes, and it has recently become attractive as a non-thermal method for bacterial inactivation in liquid foods (Kim et al., 2007b; Bae et al., 2009; Choi et al., 2009). Bacterial survival is affected both chemical and physically at relatively mild treatment conditions, preserving food quality properties (Kim et al., 2007b). Only one published work deals with *C. sakazakii* control by means of this technology (Kim, Kim and Rhee, 2010), considering its direct applicability in formula milk at the dehydrated stage. Three treatment temperatures, 63, 68 and 73 °C, were used, based on milk processing temperatures (63 °C pasteurization and 73 °C high temperature sterilization), and $C0_2$ gas (99.5 %) was injected in the system, working at 15, 20 and 25 MPa, to process 10 g of contaminated sample.

Initially a thermal study was carried out and the effect of temperature was determined, proving ineffective against *C. sakazakii* with a time of 30 min. In a second work step, the application of

SC-CO_2 in combination with the temperatures studied resulted in an increase in *C. sakazakii* inactivation levels (e.g. at 10 MPa, 30 min: 1.92 $\log_{10}$ cycles (SC-CO_2 at 63 °C); 3.76 $\log_{10}$ cycles (SC-CO_2 at 68 °C) and 6.32 $\log_{10}$ cycles (SC-$C0_2$ at 73 °C)). The effect of temperature on inactivation proved more significant than the effect of pressure. For example, at 20 MPa, 30 min, the microbial count was reduced from an initial count of 7 $\log_{10}$ to 2.5 $\log_{10}$ cycles at 63 °C; to 3.99 $\log_{10}$ at 68 °C and to undetectable limits at 73 °C; at 73 °C, 10 min, the microbial count was reduced from 7 $\log_{10}$ to 4 $\log_{10}$ cycles at 15 MPa, to 3 $\log_{10}$ cycles at 20MPa and to 2.5 $\log_{10}$ cycles at 25 MPa. The major effect of temperature has been possibly related to the diffusivity of $C0_2$, owing to the increase in cell membrane fluidity (Hong and Pyun, 1999).

Ultraviolet Light

With the aim of eliminating and controlling *C. sakazakii* in dry formula, ultraviolet (UV) treatment applied directly to powdered non-sterile products is presented as a potential alternative to reduce the risk associated with neonatal feeding. Recently, Liu et al. (2012) and Arroyo et al. (2011d) reported *C. sakazakii* resistance to ultraviolet radiation, working in a radiation range from 12.1 ± 0.30 kJ/m^2 to 72.8 ± 1.83 kJ/m^2 and light 88·55 mW/cm^2, respectively. The mechanism of UV microbial inactivation has been extensively reported as being due to formation of pyrimidine dimers that distort the DNA helix and block microbial cell replication (Miller et al., 1999). The mechanism of inactivation was evaluated by Liu et al. (2012) using Fourier transform infrared spectroscopy (FT-IR) (4000 to 400 cm^{-1}). Liu et al. (2012) used a UV chamber containing two 45.4-cm-long UV lamps suspended across the chamber. The maximum level of inactivation was 1.38 $\log_{10}$ cfu/g (Liu et al., 2012), achieved after 25 min of UV

exposure. The *C. sakazakii* survival curves obtained by Liu et al. (2012) showed an initial rapid inactivation (attributed to UV radiation exposure) in the first 5 min, followed by a second rapid inactivation after 25–30 min of UV exposure (attributed to the temperature achieved in the chamber). Complete inactivation of *C. sakazakii* in dry formula by this technology was not achieved, owing to incomplete penetration of UV radiation. Consequently, Liu et al. (2012) proposed the application of this technology as a post-pasteurization process to control *C. sakazakii*, improving the penetration radiation dose in the product. According to U.S. Food and Drug Administration (FDA) recommendations (2009), to achieve a 4-log microbial inactivation the UV radiation exposure must be at least 400 J/m^2 for all parts of the product (U.S. FDA, 2009). In the experiment carried out by Liu et al. (2012), a 25 min UV radiation treatment led to a radiation dose of about 60.7 kJ/m^2. However, UV transmittance, dose delivery, momentum transfer and consequently microbial inactivation are influenced by the optical and physical properties of powdered food and the variety of its chemical composition (Koutchma, 2008). Moreover, bacterial sensitivity to UV radiation is conditioned by cell wall structure, thickness and composition, absorbent compounds, and nucleic acid structure. All of these factors influence UV radiation effectiveness. From FT-IR examination it was concluded that spectral variations attributed to structural protein, lipids and DNA are closely related to *C. sakazakii* resistance to UV radiation.

Arroyo et al (2011d) used equipment consisting of eight individual annular thin-film flow-through reactors emitting at 254 nm and connected in series. The maximum inactivation level observed by Arroyo et al. (2011d) was around 7 $\log_{10}$ cycles for a radiation dose of ≈24 kJ/kg. Alkaline (pH 9, 1 h) and oxidative (0.5 mmol/L H_2O_2, 1 h)) pre-treatment shocks decreased *C. sakazakii* resistance to UV-C treatment, reducing the time required to achieve 3 $\log_{10}$ cycles of inactivation by 18–50 %.

Currently, sterilization systems (100 to 1000 kg/h) for powder are offered by the industry. A UV sterilization system connected after the drying step could be an effective alternative to reduce the *C. sakazakii* risk in these products specifically due to PIFM post-treatment environmental contamination.

Concluding remarks

The industrial importance of this study is related to the formulation of milk-based beverages aimed at children between 1 month and 3 years old that include powdered milk in the product formulation. Despite the advantages of cost and storage of powder over the ready-to-consume sterile liquid form, PIFM is not a sterile product, with the increasing concern about the risk of *C. sakazakii* contamination, due to severe consequences for the neonatal population. Regarding PIFM manufacture, two main steps should be noted from a microbiological inactivation point of view: (i) pasteurization (71.6 °C, 15 s) of the mixed ingredients; (ii) followed by drying with inlet air temperatures ranging from 135 to 204 °C. However, up to date, no sterilization process has been developed to guarantee PIFM safety. From our knowledge, irradiation could be an

alternative due to its applicability directly on powder products with high *C. sakazakii* inactivation levels and no-regrowth during refrigerated storage post-treatment.

In spite of the different preservation methods applied during PIFM production and reconstitution, the main source of risk has been attributed to post-processing PIFM recontamination (FAO/WHO, 2004). Therefore, in addition, and according to Codex specifications and FAO/WHO (2006) recommendations, reconstitution of PIFM should be performed with hot water above 70 °C, achieving *C. sakazakii* inactivation levels around 5 $\log_{10}$ cycles. Given the great importance of the nutritional quality and sensorial properties of infant formula, owing to the special requirements of infants and children (0–3 years), some public health organizations point out the possible nutritional quality reduction due to thermal PIFM reconstitution (Agostini et al., 2004; FAO/WHO, 2006). Consequently, alternative methods to thermal pasteurization, sterilization, and reconstitution of PIFM are being sought to generate microbiologically safe liquid and powder formulas with added value.

This paper aims to provide an overview of the application of non-thermal technologies to control *C. sakazakii* in RPIFM, and to detect future infant feeding preservation strategies in the manufacture and handling of PIFM and reconstituted PIFM, for example, in hospital settings. The application of non-thermal technologies to *C. sakazakii* inactivation, reported and summarized in this paper, has been undertaken since the year 2001 (Table 1), with research indicating the current worldwide interest in the subject, in response to International Health Organization calls to process RPIFM using alternative non-thermal methods. All of the non-thermal technologies studied are able to achieve complete inactivation of *C. sakazakii*, [2–9] $\log_{10}$ cycles, given the low levels of prevalence found in PIFM (less than 1 cfu/g) (FAO/WHO,

2008). The inactivation level achieved depends on the technology, treatment conditions, substrate and bacterial strain. Differences between authors might be attributable to equipment and non-standardized treatment conditions. However, all of these non-thermal technologies are still in an early stage of development with regard to their application in the production of PIFM while ensuring the microbiological safety of the product. The most promising technologies, both from a food safety and practical point of view, to be directly applied on PIFM or RPIFM, are ranked as follows: high hydrostatic pressure > gamma irradiation > pulsed electric fields> ultraviolet light. In order to validate the matching of product and technology, the next step would be to scale up to industrial level. A better understanding of the complex mechanism of action of non-thermal processing technologies and their effects on microorganisms (adaptation of bacterial response by modifying the proteomic profile, generation of sublethal damage, increased virulence or bacterial sensitization to subsequent stress) and shelf life studies are still needed to fill gaps in knowledge related to the application of non-thermal technologies to *C. sakazakii* inactivation.

In recent years, special attention has been paid to hurdle technology as a future potentially effective method for treating IFM at non-intensive treatment conditions, largely preserving the nutritional quality of the product and preserving food safety. In this sense, and according to Pina-Pérez et al. (2011a, 2012), rich in polyphenols-cocoa bacteriostatic effect could be used in novel infant beverages formulation, with possible *C. sakazakii* inactivation synergistic or additive effects in combination with other non-thermal pasteurization technologies under "hurdle concept". The cocoa use combined with PEFs treatment and refrigerated storage (up to 8ºC) seems to be a potential pasteurization method to be applied on future rich-in-polyphenols cocoa

infant beverages with antimicrobial synergistic effects against *C. sakazakii*, being involved in a patentability process (Martinez, Rodrigo, and Pina-Pérez, 2011b)

It can be foreseen that a deeper understanding of (i) the most effective application for each non-thermal technology, separately or jointly, achieving food safety and quality aims; (ii) the resistance of *C. sakazakii* to non-thermal technologies to be applied in manufacturing processes and hospital feeding services; (iii) the capability of the microorganism to adapt and respond to treatment and subsequent conditions, will provide key tools for the development of novel infant beverages processed by new technologies and the implementation of new strategies to improve the microbiological stability and quality of these formulas.

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Table 1 Despite the potential of the results obtained, knowledge remains scarce and more confirmatory studies are required to cover food quality and safety aspects for each technology application in infant formula milk to avoid risks associated with this emerging pathogen. The focus of this review is (i) the importance of the application of non-thermal technologies for the control of *C. sakazakii* as part of the development of strategies in the context of improving PIFM and RPIFM food safety and quality.

Table1. Summary of inactivation levels achieved by non-thermal technologies applied to *Cronobacter sakazakii* control

Technology	References	Treatment conditions	Inactivation levels	Substrate
High Hydrostatic Pressure	González et al. 2006	600 MPa/1 min	3 $\log_{10}$ (ATCC 29544)	RPIFM
Processing (HPP)		600 MPa/1 min	6 $\log_{10}$ (ATCC 12868)	RPIFM
	Pina-Pérez et al. 2007a	100-400 MPa/1-20 min	2-7 $\log_{10}$	RPIFM
	Koseki, Matsubara & Yamamoto 2009	400-600 MPa /1-20 min	5 $\log_{10}$	RPIFM
	Arroyo et al. 2011a	100-600 MPa	5-7 $\log_{10}$	RPIFM
Pulsed Electric Fields (PEF)	Pina-Pérez et al. 2007b	10-40 kV/cm/60-3000 µs	Maximum 1.20 $\log_{10}$	RPIFM
	Arroyo et al. 2010a	50 pulses at 31 kV/cm	Maximum 5 $\log_{10}$	CPB
	Pina-Pérez, Rodrigo, Martinez, 2009	15-35 kV/cm/60-3000 µs	Maximum 1.22 $\log_{10}$	RPIFM
Ultrasound waves (UW)	Adekunte et al. 2010	24-61 µm/25-50 °C	≈7 $\log_{10}$	RPIFM
	Arroyo et al. 2011b, 2011c	56 °C/117 µs, 200 kPa, 1.5 min	3.2 $\log_{10}$	CPB
Gamma irradiation	Lee, Kermasha, & Baker, 2007	5 kGy dose	8-9 $\log_{10}$	PIFM
	Osaili et al. 2007	8 KGy dose	7 $\log_{10}$	PIFM
Electron beam irradiation	Hong et al. 2008	16 kGy	7 $\log_{10}$	RPIFM
Antimicrobials				
Monocaprylin	Nair, Joy, & Venkitanarayanan, 2004	0.25-50 mM/4-37 °C	2-6 $\log_{10}$	RPIFM
Caprylic acid	Jang and Rhee, 2009	5, 10, 20, 30 Mm/25 °C	7.7 $\log_{10}$	RPIFM
Trans-cinnamaldehyde	Amalaradjou, Hoagland and Venkitanarayanan, 2009	0-0.5 % (w/v) / 4-23 °C	5-7 $\log_{10}$	RPIFM
Rich in polyphenols cocoa	Pina-Pérez, Rodrigo, Martinez,	1-5 % (w/v)	Bacteriostatic effect	RPIFM

	2011			
Bovine lactoferrin (LF)	Al-Nabulsi et al. 2009	2.5 mg/mL/1h/37 °C	4 $\log_{10}$	0.2 % PW
	Al-Nabulsi et al. 2009	2.5-10 mg/mL/21-37 °C	No inact. observed	RPIFM
Nisin	Al-Nabulsi et al.2009	1500 IU/mL/21-37 °C	4 $\log_{10}$	0.2 % PW
	Al-Nabulsi et al. 2009	500-2000 IU/mL	No inact. observed	RPIFM
Carvacrol, thymol, eugenol, diacetyl, cinnamic acid	Lee and Jin, 2008	MICs: 1.25 mmol/L to 5 mmol/L	Growth inhibition	TSB
Muscadine seed extracts	Kim et al. 2009	muscadine seed powders/30-60 min	> 7 $\log_{10}$	TSB
Copper sulphate	Al-Holy, Castro, & Al-Qadiri, 2010	50 ppm/2 h-6 h	3 $\log_{10}$ – up to 7 $\log_{10}$	RPIFM
Lactic acid	Al-Holy, Castro, & Al-Qadiri, 2010	0.2 % (v/v)/2 h-6 h	3 $\log_{10}$ – up to 7 $\log_{10}$	RPIFM
Sodium caseinate fermentate	Hayes et al. 2009	3.3 % (w/v)/ 60 min	6 $\log_{10}$	RPIFM
Non-thermal Plasma (NTP)	Joshi et al. 2010	Compressed air and helium gas/5-30 s Flow rate 4, 6, 8 Lpm	0.12-6.94 $\log_{10}$	RPIFM
Bacteriophages	Kim . Klumpp & Loessner, 2007	24 °C, 2h, 10^7-10^9 PFU/mL ESP 1-3; ESP 732-1	8 $\log_{10}$	BHI
	Zuber et al. 2008	10^8 PFU/mL	>6 $\log_{10}$	Broth
Supercritical carbon dioxide	Kim, Kim and Rhee, 2010	CO_2 gas (99.5 %); [15-25] MPa; 63-73 °C	2-6 $\log_{10}$	PIFM
Ultraviolet light	Arroyo et al. 2011d	24 kJ/kg	7 $\log_{10}$	CPB
	Liu et al. 2012	60.7 kJ/m^2 (25min) irradiation dose	1.38 $\log_{10}$	PIFM

RPIFM: Reconstituted powdered infant formula milk; CPB: Citrate-phosphate buffer; PIFM: Powdered infant formula milk;

PW: peptone water; TSB: Tryptic Soy Broth; BHI: Brain Heart Infusion